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EFECTOS DE TWEAK DEPENDIENTES DE NF-κB EN LA CÉLULA TUBULAR RENAL

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EFECTOS DE TWEAK DEPENDIENTES DE NF-κB EN LA CÉLULA TUBULAR RENAL

Memoria que presenta la licenciada en Biología

María Concepción Izquierdo Carnero

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CERTIFICAN

Que Doña María Concepción Izquierdo Carnero, Licenciada en Ciencias Biológicas por la Universidad Complutense de Madrid, ha realizado bajo su dirección el trabajo titulado "Efectos de TWEAK dependientes de NF-κB en la célula tubular renal" que presenta como Tesis Doctoral para alcanzar el grado de Doctor por la Universidad Autónoma de Madrid.

Y para que conste, firmamos la presente en Madrid a 1 de Septiembre de 2012

Los directores de tesis,

Dr. Alberto Ortiz Arduán

Dr. Jesús Egido de los Rios

Dra. Ana Belén Sanz Bartolomé

VºBº El Tutor,

Dr. Luis del Peso

A mi mami

“La ciencia es el alma de la prosperidad de las naciones y la fuente de vida de todo progreso"

Louis Pasteur

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RESUMEN

Durante el fracaso renal agudo parenquimatoso se produce un proceso inflamatorio que intenta reparar el daño tisular, pero que a su vez, puede dañar al riñón.

Mediante transcriptómica renal identificamos nuevos mediadores del fracaso renal agudo relacionados con la inflamación que pueden contribuir a diseñar nuevas estrategias terapéuticas. En concreto, encontramos expresadas diferencialmente moléculas reguladas por TWEAK, una citoquina multifuncional que contribuye a la inflamación renal y al fracaso renal agudo *in vivo*. TWEAK activa al receptor Fn14.

El análisis transcripcional del fracaso renal agudo experimental con inflamación túbulointersticial reveló un aumento significativo de la expresión de CXCL16, una quimioquina transmembrana, que se correlacionó con la expresión de Fn14. TWEAK aumentó la expresión de CXCL16 en células tubulares tanto *in vivo* como *in vitro* de manera dependiente de NF-κB. TWEAK también aumentó la infiltración de linfocitos T *in vivo*. CXCL16 tubular aumentó en un modelo de fracaso renal agudo nefrotóxico y el bloqueo de TWEAK disminuyó la expresión de CXCL16 y la infiltración de linfocitos. En biopsias de riñón humano con inflamación tubulointersticial, la expresión de CXCL16 y Fn14 en las células tubulares se asoció con infiltrados inflamatorios. CXCL16 colaboró con TWEAK en promover una respuesta inflamatoria en células tubulares cultivadas. Sin embargo, CXCL16 no moduló ni la proliferación ni la supervivencia de la célula tubular. En conclusión, TWEAK regula la expresión de la quimioquina CXCL16 en el epitelio tubular y esto puede contribuir a la inflamación tubulointersticial renal.

El análisis del array transcripcional también mostró una disminución significativa de la expresión de Klotho, una proteína con propiedades antienvejecimiento expresada por las células renales. Además, la expresión de Klotho estaba inversamente correlacionada con la expresión de Fn14. En el marco del fracaso renal agudo inducido por ácido fólico, el bloqueo o ausencia de TWEAK evitó la disminución de los niveles renales y plasmáticos de Klotho. TWEAK disminuyó la expresión renal de Klotho tanto *in vivo* como *in vitro* de manera dependiente de NF-κB RelA. TWEAK promovió la deacetilación del promotor de Klotho. Estos datos pueden explicar parcialmente la relación entre la inflamación y las enfermedades que se caracterizan por envejecimiento acelerado de los órganos, incluyendo enfermedad renal crónica.

SUMMARY

Acute kidney injury is associated to an inflammatory process that attempts to repair tissue damage, but that may cause further damage to the kidney.

Renal transcriptomics identified novel inflammatory mediators of acute kidney injury that can contribute to design new therapeutic strategies. Specifically, we found differential expression of molecules regulated by TWEAK, a multifunctional cytokine that contributes to renal inflammation and acute kidney injury in vivo. TWEAK activates the Fn14 receptor.

Transcriptional analysis of experimental acute kidney injury with tubulointerstitial inflammation revealed a correlation between an upregulation of the expression of CXCL16, a transmembrane chemokine, and Fn14 expression. TWEAK increased kidney tubular cell CXCL16 expression both in vivo and in vitro in a NF-κB dependent manner. TWEAK also increased T-lymphocyte infiltration in vivo. Tubular cell CXCL16 was increased in a nephrotoxic acute kidney injury model and TWEAK blockade decreased CXCL16 expression and lymphocyte infiltration. In human kidney biopsies with tubulointerstitial inflammation, tubular cell CXCL16 and Fn14 expressions were associated with inflammatory infiltrates. In cultures tubular cells CXCL16 appeared to synergize with TWEAK to promote an inflammatory response. However, it did not modulate tubular cell proliferation or survival. In conclusion, TWEAK upregulates the expression of the chemokine CXCL16 in tubular epithelium and this may contribute to kidney tubulointerstitial inflammation.

Transcriptional analysis also showed a downregulation of Klotho expression. Klotho is a protein expressed by renal cells with antiaging properties. Furthermore, Klotho expression was inversely correlated with Fn14 expression. In the setting of acute kidney injury induced by folic acid, the blockade or absence of TWEAK abrogated the decrease in renal and plasma Klotho levels. TWEAK reduced renal Klotho expression both in vivo and in vitro in an NF-κB RelA dependent manner. TWEAK promoted deacetylation of promoter Klotho. These data may partially explain the relationship between inflammation and diseases characterized by accelerated aging, including chronic kidney disease.

ÍNDICE

ABREVIATURAS

aa	AMINOÁCIDOS
ADAM	DESINTEGRINA Y METALOPROTEINASA
ADN	ÁCIDO DESOXIRRIBONUCLEICO
ADNc	ÁCIDO DESOXIRRIBONUCLEICO COMPLEMENTARIO
AMPc	ADENOSIN MONOFOSFATO CÍCLICO
ARN	ÁCIDO RIBONUCLEICO
ARNm	ÁCIDO RIBONUCLEICO MENSAJERO
DHR	DOMINIO DE HOMOLOGÍA REL
ERC	ENFERMEDAD RENAL CRÓNICA
FGF	FACTOR DE CRECIMIENTO DE FIBROBLASTOS
Fn14	FACTOR INDUCIBLE POR FACTORES DE CRECIMIENTO DE FIBROBLASTOS-14
FRA	FRACASO RENAL AGUDO
HDAC	HISTONA DEACETILASA
HUVEC	CÉLULAS ENDOTELIALES UMBILICALES HUMANAS
ICAM-1	MOLÉCULA DE ADHESIÓN INTRACELULAR
IFN-γ	INTERFERON- Γ
IKK	QUINASA DE IKB
IL	INTERLEUQUINA
LDLox	LIPOPROTEÍNAS DE BAJA DENSIDAD OXIDADAS
LPS	LIPOPOLISACÁRIDO BACTERIANO
MCP-1	PROTEÍNA QUIMIOTÁCTICA DE MACRÓFAGOS
mTWEAK	TWEAK DE MEMBRANA
ND	FROPATÍA DIABÉTICA
NF-κB	FACTOR NUCLEAR-ΚB
NIK	QUINASA INDUCTORA DE NF-ΚB
NL	NEFRITIS LÚPICA
NM	NEFROPATÍA MEMBRANOSA
NTA	NECROSIS TUBULAR AGUDA
PKC	PROTEÍNA QUINASA C
siRNA	ARN DE SILENCIAMIENTO
SLN	SEÑAL DE LOCALIZACIÓN NUCLEAR
sTWEAK	TWEAK SOLUBLE
TNF	FACTOR DE NECROSIS TUMORAL

TNFR1	RECEPTOR 1 DE TNF
TNFR2	RECEPTOR 2 DE TNF
TNFRSF	RECEPTORES DE LA SUPERFAMILIA DEL TNF
TNFSF	SUPERFAMILIA DEL TNF
TWEAK	INDUCTOR DÉBIL DE APOPTOSIS SIMILAR AL FACTOR DE NECROSIS TUMORAL
TWEAKR	RECEPTOR DE TWEAK
UUO	OBSTRUCCIÓN UNILATERAL DEL URÉTER

I. INTRODUCCIÓN

1. EL RIÑON Y EL TÚBULOINTERSTICIO RENAL. FISOPATOLOGÍA

1.1. El riñón

El riñón es un órgano vital que realiza funciones fundamentales para el mantenimiento de la homeostasis del organismo:

- Eliminación de desechos del metabolismo en forma de orina.
- Mantenimiento del equilibrio hidroelectrolítico y ácido-base.
- Endocrino-metabólica, destacando la producción de Eritropoyetina.

Los riñones eliminan la urea y otros productos desechables de la sangre a través de unas unidades de filtración denominadas nefronas. La nefrona constituye la unidad estructural y funcional de los riñones. Cada nefrona está formada por el corpúsculo renal o corpúsculo de Malpighi (formado a su vez por el glomérulo y la cápsula de Bowman), el túbulo contorneado proximal, los segmentos delgado y grueso del asa de Henle y el túbulo contorneado distal. Los corpúsculos de Malpighi y los túbulos proximal y distal pertenecen a la zona denominada corteza, mientras que los dos segmentos del asa de Henle se localizan en la médula (**Figura 1**).

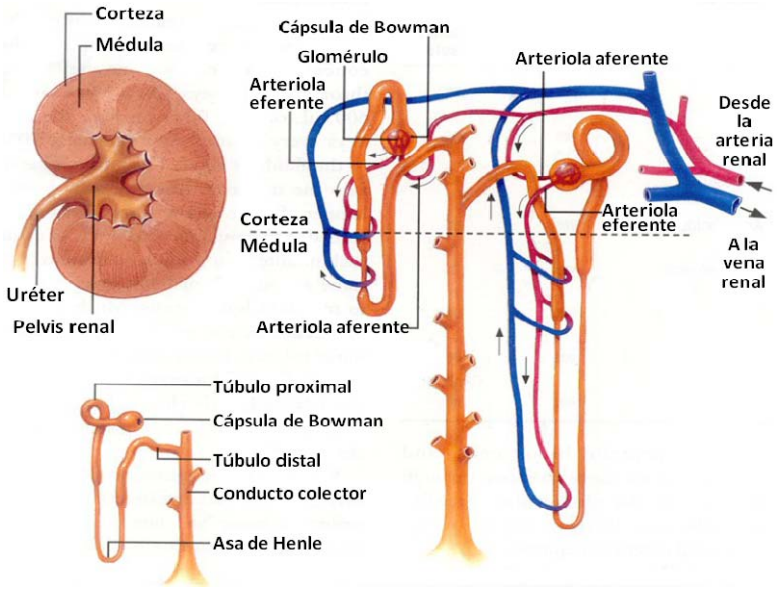

Figura 1. ***Estructura del riñón.*** *En la parte superior izquierda, corte esquemático de riñón que representa la topografía general del órgano. A la derecha, localización cortical y medular de los componentes de la nefrona y del sistema de los conductos colectores. En la parte inferior izquierda, esquema simplificado de la nefrona.*

1.2. El daño renal

Existen varios tipos de nefropatías que se clasifican según la porción del riñón que se ve afectada con más intensidad (glomerulares, túbulointersticiales, vasculares). El daño renal puede ser agudo o crónico. La forma más frecuente de fracaso renal agudo (FRA) parenquimatoso es la lesión tubular. Además, la pérdida progresiva de la función renal se correlaciona con el daño tubular, independientemente del origen de la enfermedad [224]. La inflamación es un componente fundamental

del daño renal [224]. Se genera como respuesta a la agresión, pero, a su vez, puede dañar al riñón. Cuando la inflamación es exacerbada en intensidad y tiempo, acaba produciendo un daño crónico que merma la función renal para siempre. Las células tubulares renales juegan un papel importante en la inflamación [51;79] y producen citoquinas, quimioquinas y moléculas de adhesión [288] que atraen células inflamatorias al riñón. La enfermedad renal crónica (ERC) predispone al FRA y el FRA agrava la ERC. Sin embargo, el arsenal terapéutico del nefrólogo en el FRA o la ERC es pobre. Por estas razones hemos centrado estos estudios en la búsqueda de alternativas terapéuticas para la lesión renal relacionadas con la inflamación y con las células tubulares renales.

1.3. El fracaso renal agudo

Independientemente del tipo de nefropatía, la consecuencia más temida del daño renal es el fracaso de las funciones del riñón, que puede ocasionar la muerte del paciente. El FRA es una pérdida brusca de la función renal. La causa más frecuente de FRA parenquimatoso es la necrosis tubular aguda [142]. El término de necrosis tubular aguda fue creado antes de que se definiera la apoptosis como una forma distinta de muerte celular. Sin embargo, en la necrosis tubular aguda humana la forma más frecuente de muerte celular es la apoptosis [201;264]. Los cambios morfológicos del FRA en el ser humano incluyen lesión y muerte celular, regeneración, inflamación y edema intersticial [201]. Las opciones del nefrólogo para el tratamiento de la necrosis tubular aguda se limitan a sustituir la función renal mediante diálisis si es preciso, y no existen maniobras que permitan acelerar la recuperación funcional del riñón. En este sentido la mortalidad del FRA permanece estable, en torno al 50%, desde hace 40 años. Los riñones tienen la capacidad de recuperarse espontáneamente de un daño agudo si el paciente sobrevive.

Durante el FRA, hay una primera fase de muerte de las células tubulares, seguido de una fase de regeneración celular y recuperación [114]**.** Las células tubulares que permanecen en los túbulos después del daño proliferan para regenerar el epitelio tubular y recuperar la función renal. Las células madre también participan en la reparación tisular después de un daño [122]. Recientemente se ha sugerido la existencia de células progenitoras en el riñón que participan en la reparación del epitelio tubular después de un daño. Estas células proliferarían después de un daño renal y se diferenciarían para contribuir a la regeneración tubular [160].

Los modelos experimentales de FRA permiten abordar su patogenia en busca de nuevas alternativas terapéuticas. Una sobredosis de ácido fólico induce el FRA en los seres humanos [170] y en los ratones [203;207]. El FRA experimental inducido por ácido fólico comparte con el FRA humano la presencia de apoptosis en las células tubulares, la proliferación compensatoria de estas células que conduce a la regeneración, la infiltración de células inflamatorias y, en una fase crónica, la fibrosis leve [203;207]. En el

FRA por ácido fólico [60] o por cisplatino están aumentados un gran número de citoquinas y quimioquinas que facilitan el infiltrado de leucocitos en el riñón [222].

2. BÚSQUEDA DE BIOMARCADORES Y DIANAS TERAPÉUTICAS MEDIANTE TÉCNICAS DE ALTO RENDIMIENTO

Tradicionalmente, la búsqueda de mediadores de daño tisular entrañaba una revisión cuidadosa de la literatura a la búsqueda de nuevas moléculas con características que pudieran ser relevantes en la lesión renal. Con este abordaje teórico se identificaban moléculas de interés potencial y se estudiaba su expresión en la lesión. Si existían diferencias entre el tejido normal y el dañado, se procedía a un análisis funcional en células cultivadas y en modelos animales. Este proceso es poco eficaz y consume muchos recursos. En 1986, Thomas Roderick definió el término "*genómica*" para describir las diferentes disciplinas de mapeo, secuenciación y análisis del genoma (conjunto de genes de un organismo). A finales de los 90 se completaron los primeros genomas de organismos eucariotas siendo la culminación de todos los estudios la secuenciación del genoma humano[289]. La época post-genómica ha desarrollado una serie de metodologías experimentales destinadas a estudiar la información derivada de las secuencias genómicas para caracterizar su funcionalidad biológica. Es lo que se denomina: "*genómica funcional*". Actualmente la genómica funcional se encuentra dividida en cuatro ramas: el conocimiento del genoma, del transcriptoma (conjunto de moléculas de ARNm), del proteoma (conjunto de proteínas) y del metaboloma (conjunto de metabolitos de bajo peso molecular).

2.1. Transcriptómica. Tecnología de microarrays

La transcriptómica es una técnica de alto rendimiento que permite la cuantificación de la expresión de miles de genes en base a su expresión diferencial en la patología.

Las técnicas basadas en chips de ADN (*microarrays*) permiten monitorizar el transcriptoma de un modo rápido y reproducible[43].

La transcriptómica implica el manejo simultáneo de miles de datos, lo que se logra mediante herramientas bioinformáticas y bioestadísticas. Estas técnicas implican un control de calidad de los datos con normalizaciones para eliminar la variabilidad sistémica introducida por el proceso técnico. La metodología del análisis pretende buscar genes diferencialmente expresados entre las diferentes muestras.

En esta tesis hemos utilizado un abordaje transcriptómico como método para identificar nuevos genes implicados en el FRA que pudieran servir como biomarcadores o dianas terapéuticas. En concreto nos hemos centrado en 2 genes, CXCL16 y Klotho cuya expresión fue significativamente diferencial entre las muestras de FRA y los riñones control, y se correlacionan bien con la expresión de Fn14, el

receptor de TWEAK. Nuestro laboratorio tiene una larga experiencia en el estudio del papel de TWEAK en la patogenia del daño renal [210;243;248].

3. SUPERFAMILIA DEL TNF

El factor de necrosis tumoral (TNF) fue aislado y clonado hace 28 años [3;216]. Esta molécula se convirtió en el prototipo de una familia de proteínas con características en común conocidas como la superfamilia del TNF (TNFSF). La mayoría de los miembros de esta gran familia se sintetizan como proteínas transmembrana de tipo II y comparten un motivo estructural común, el dominio de homología TNF, que interviene en la auto-trimerización y en la unión al receptor [18;85]. El dominio extracelular puede ser escindido por proteasas específicas generando citoquinas solubles. La unión de los ligandos de la TNFSF a sus receptores juega un papel crucial en muchos procesos biológicos fundamentales, incluyendo la apoptosis, la diferenciación celular, y la inflamación [27]. Muchas de las acciones de esta superfamilia están relacionadas con la activación del factor de transcripción NF-κB.

Algunas de estas citoquinas, como TNF o TWEAK han sido estudiadas en el riñón y están involucradas en el daño renal [125;150;173].

3.1. TNFα

TNFα es una potente citoquina capaz de producir y controlar la respuesta inflamatoria, además de inducir apoptosis en una gran variedad de tipos celulares y tejidos [236;273]. En el riñón, TNFα se expresa, se sintetiza y se libera por los macrófagos infiltrantes y las células renales intrínsecas, como las células endoteliales, mesangiales, glomerulares y las células epiteliales tubulares [206]. TNFα activa dos receptores, TNFR1 y TNFR2. TNFR1 está presente en el glomérulo normal y sus niveles aumentan en respuesta al daño renal, mientras que TNFR2 no se expresa en el riñón normal y aparece en las células tubulares en respuesta al daño renal [65]. TNFα activa las células renales, induciendo la producción y liberación de factores quimiotácticos, la expresión de moléculas de adhesión y el desarrollo de la actividad procoagulante. Estudios *in vivo* demuestran que el TNFα posee efectos citotóxicos frente a células glomerulares, mesangiales y epiteliales**.** Se ha demostrado, el papel patogénico de TNFα, así como los beneficios potenciales de la modulación de la actividad del TNFα en modelos de glomerulonefritis mediada por inmuno-complejos, nefritis lúpica, glomerulonefritis asociada a anticuerpos citoplásmicos antineutrofilos, enfermedad de cambios mínimos, nefropatía diabética (ND), FRA, uropatía obstructiva, y el rechazo del injerto renal [6;47;64;65;173;192;206;222]. La deficiencia de TNFR1 o de TNFR2 protege a los ratones del FRA inducido por cisplatino [223;284] y de la uropatía obstructiva [88]. TNFα también tiene funciones inmunosupresoras, dependiendo del entorno circundante, el momento de la respuesta inflamatoria, y la interacción diferencial con sus receptores [65]. Cada vez hay más evidencias

que sitúan al TNFα como un elemento clave en la patogénesis de la lesión renal, promoviendo la inflamación, la apoptosis y la acumulación de matriz extracelular [62;84;130;173;192;206;209].

3.2. TWEAK

El factor inductor débil de apoptosis similar al factor de necrosis tumoral (TWEAK) es una citoquina de la superfamilia del TNF que activa al receptor Fn14 (factor inducible por factores de crecimiento de fibroblastos-14) y que fue identificada en 1997 [39].

3.2.1. Estructura de TWEAK

TWEAK humano es una glicoproteína transmembrana de tipo II de 249 aminoácidos (aa). Está formada por un dominio C-terminal extracelular de 206-aa que contiene el sitio de unión al receptor y un sitio potencial de N-glicosilación [39], un dominio transmembrana de 25-aa y un dominio N-terminal intracelular de 18-aa (**Figura 2**). La mayoría de las células expresan dos isoformas de TWEAK, una forma anclada a la membrana (mTWEAK) y otra forma soluble (sTWEAK) que se forma por el procesamiento de la isoforma anclada a la membrana [30;299]. Los genes humano y murino de TWEAK presentan una similitud del 93% en la secuencia de unión al receptor. Así pues, tanto sTWEAK como mTWEAK humanos se unen al receptor Fn14 murino con la misma afinidad que al Fn14 humano [22].

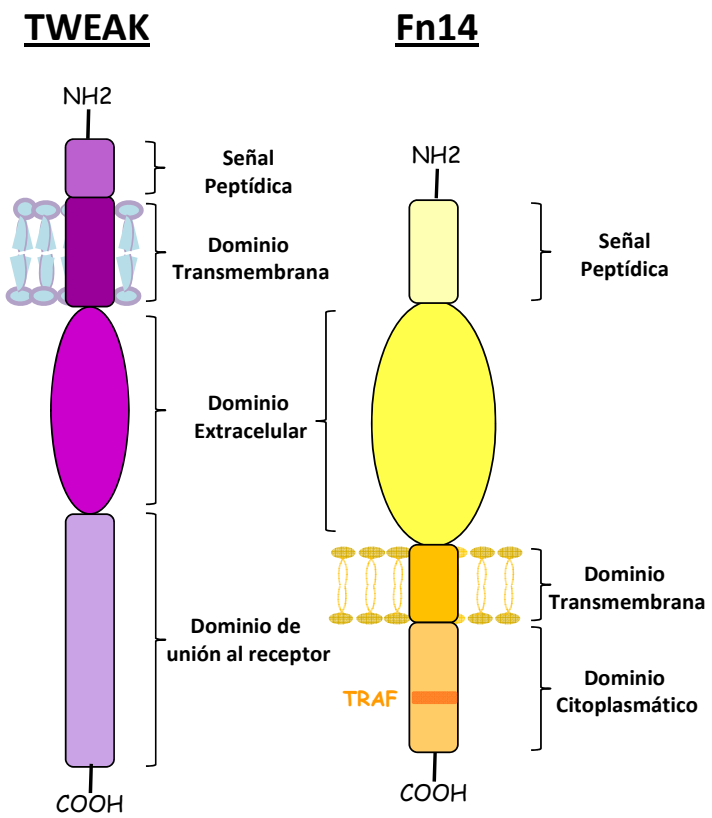

Figura 2: ***Estructura de la citoquina TWEAK y de su receptor, Fn14.***

3.2.2. El receptor de TWEAK: Fn14

En 2001 se publicó un artículo que identificaba un nuevo receptor para TWEAK al que los autores denominaron receptor de TWEAK (TWEAKR)[297]. TWEAKR era idéntico a Fn14. Fn14 había sido descrito previamente como el producto de un gen inducible por factores de crecimiento en fibroblastos murinos [166]. Los homotrímeros de TWEAK no se unen a otros miembros de la familia de receptores de TNF (TNFRSF) y otros homotrímeros de la familia TNF no pueden asociarse al receptor Fn14 [297]. Fn14 es una proteína transmembrana de tipo I con 129 aminoácidos de peso molecular 13,911 KDa [68;297]. Cuando se procesa es el miembro más pequeño de la TNFRSF (**Figura 2**). Los receptores Fn14 humano y murino presentan una similitud del 90%, y ambos tienen características estructurales típicas de la TNFRSF. En 2007 se publicó que TWEAK se une también a CD163 [23]. CD163 es un receptor basurero de expresión restringida a monocitos macrófagos, que es responsable de aclarar la circulación los complejos haptoglobina-hemoglobina. CD163 podría actuar como un receptor basurero para TWEAK en situaciones patológicas [23].

3.2.3. Expresión de TWEAK

TWEAK se expresa en numerosos tejidos y se puede encontrar a niveles elevados en el páncreas, intestino, corazón, cerebro, pulmón, ovario, sistema vascular y músculo esquelético, y a niveles más bajos en el hígado y el riñón [39]. Sin embargo, las fuentes celulares precisas del sTWEAK circulante no están claras. Los mecanismos que regulan la expresión de TWEAK no son muy conocidos. La expresión de TWEAK disminuye en modelos de inflamación aguda o crónica en numerosos tejidos y en macrófagos peritoneales [41]. En cambio, en otros modelos inflamatorios, como es la encefalitis autoinmune experimental, los niveles de TWEAK están elevados [57]. Tanto TWEAK como Fn14 se expresan en el riñón. En los primeros trabajos se sugería que las fuentes potenciales de TWEAK en el riñón eran monocitos/macrófagos y células T infiltrantes, en particular células T de pacientes con lupus [128;187]. Sin embargo, tenía que haber otras células intrínsecas renales que expresasen TWEAK, ya que la citoquina se expresaba en el riñón sano. Hasta ahora, además de los estudios de nuestro grupo que demuestran que TWEAK se expresa en el túbulo renal, se ha descrito que las células mesangiales también expresan TWEAK y Fn14 [29] y que los podocitos expresan al menos el receptor [74].

3.2.4. Actividad biológica de TWEAK

TWEAK tiene múltiples funciones con relevancia fisiopatológica que dependen del microambiente, del tipo de célula y del estado de activación celular. TWEAK puede regular la muerte, proliferación, migración y diferenciación celular, la inflamación, la regeneración de tejidos y la neoangiogénesis [122;154;156;176;185;191;196;277;282;291]. A continuación resumimos la información disponible sobre las funciones de TWEAK en células renales.

Apoptosis

TWEAK se describió como una citoquina con una débil capacidad para inducir la apoptosis. Así se requieren períodos largos de incubación y coestimulación con agentes sensibilizantes como interferon (IFN)γ para inducir la apoptosis [187].

En el riñón, TWEAK es capaz de inducir apoptosis de forma débil en células mesangiales, y este efecto se magnifica si se preestimulan con IFNγ [29]. TWEAK no induce apoptosis en células tubulares no estresadas pero sí cuando activa Fn14 en un ambiente inflamatorio formado por la combinación de TNFα e IFNγ [126]. A través de este mecanismo TWEAK podría facilitar la apoptosis tubular durante el FRA ya que la expresión de TWEAK, TNFα e IFNγ está incrementada en esta patología [126].

Proliferación

La proliferación inducida por TWEAK es dependiente del tipo celular [57;63;154;196;279]. En el riñón, TWEAK también induce la proliferación de células mesangiales, podocitos y células tubulares proximales humanas y murinas. Este efecto de TWEAK en el riñón está mediado por la unión a su receptor Fn14 y por el factor de transcripción NF-κB [126;244].

Tanto el suero como las citoquinas inflamatorias aumentan la expresión de Fn14 pero facilitan respuestas opuestas a TWEAK: proliferación y apoptosis, respectivamente [244].

Inflamación

Existen múltiples indicios que implican a TWEAK como mediador de la inflamación. En el riñón, se ha estudiado en detalle el efecto pro-inflamatorio de TWEAK en las células tubulares renales [50;242;288]. El factor de transcripción NF-κB juega un papel clave en la respuesta inflamatoria de las células tubulares [249]. La unión de TWEAK a Fn14 activa rápidamente la vía canónica de NF-κB [242]. Así, TWEAK induce la traslocación nuclear de RelA sensible a partenolide y la unión de los complejos de RelA al ADN, lo que permite la expresión génica y la secreción de MCP-1, IL-6 y RANTES [242;246]. Por otro lado, TWEAK puede activar la vía no canónica de NF-κB (RelB/NF-κB2) de manera más tardía induciendo la expresión de CCL21 y CCL19 [232;246]. Además, TWEAK tiene un efecto sinérgico con las lipoproteínas de baja densidad oxidadas (LDLox) en promover la expresión de genes inflamatorios en las células tubulares [183]. TWEAK también estimula la producción de MCP-1, RANTES, CXCL10 y CXCL1 en células mesangiales cultivadas y en podocitos [29;74].

El efecto proinflamatorio de TWEAK en el riñón también se observa in vivo. La administración sistémica de TWEAK induce la activación de las vías canónica y no canónica de NF-κB en las células tubulares renales, la expresión tubular de quimioquinas e infiltración intersticial por macrófagos y células T [29;74;242;246]. Los ratones deficientes de TWEAK presentan menos infiltrado de macrófagos tras un FRA inducido por sobredosis de ácido fólico [242].

3.2.5. TWEAK en el daño renal experimental y humano

La expresión de TWEAK y Fn14 está modulada en el daño renal. Fn14 aumenta en el riñón en el FRA experimental y clínico [107;126]. TWEAK renal aumenta en el FRA experimental, pero disminuye en la nefritis lúpica (NL) experimental [42;126]. TWEAK aumenta en leucocitos de sangre periférica y en orina de pacientes con lupus [146;255].

TWEAK en el fracaso renal agudo

La expresión tubular de TWEAK y Fn14 aumenta en el FRA experimental inducido por ácido fólico [126].

El bloqueo de TWEAK in vivo permite estudiar su función en el microambiente del FRA. Dos enfoques diferentes, los anticuerpos neutralizantes anti-TWEAK o ratones knock-out (TWEAK KO) protegieron del FRA inducido por ácido fólico conservando la función renal y disminuyendo la apoptosis tubular, la expresión tubular de quimioquinas (MCP-1, RANTES, y CCL21) y la inflamación intersticial por macrófagos y linfocitos T [242;244 242;246]. Un enfoque adicional, el bloqueo de Fn14, también disminuyó la lesión tubular en otro modelo diferente de FRA, inducido por isquemia-reperfusión renal donde se redujo la apoptosis de las células renales y la inflamación [107]. La infiltración de células inflamatorias en el FRA depende de la expresión local de citoquinas inflamatorias [51;288].

En resumen, el bloqueo preventivo de TWEAK o Fn14 protege del FRA experimental.

4. FACTOR DE TRANSCRIPCIÓN NF-κB

El factor nuclear (NF)-κB es una familia de factores de transcripción pleiotrópicos que controlan e integran una compleja red de estímulos extracelulares y vías de señalización, que regulan transcripcionalmente cientos de genes dependiendo del contexto, el estímulo y el sistema celular [13;98;103;217;290]. El NF-κB es un homodímero o heterodímero formado por los miembros de la familia de proteínas Rel, que en mamíferos está representada por cinco integrantes: c-Rel, NF-κB1 (p50, que se genera a partir de p105), NF-κB2 (p52 que se genera a partir de p100), RelA (p65) y RelB. Todas estas proteínas presentan una región muy conservada de aproximadamente 300 aminoácidos en el extremo N-terminal, denominada dominio de homología Rel (DHR) [249]. Dicha región es la responsable de la unión al ADN, la dimerización y la interacción con los miembros de la familia de proteínas inhibitorias IκB. El DHR contiene una señal de localización nuclear (SLN) que facilita la translocación del NF-κB al núcleo.

La regulación de la vía de NF-κB implica a unas proteínas inhibitorias, llamadas proteínas IκB, que permanecen unidas a los dímeros de NF-κB evitando que se transloquen al núcleo. Esta familia está integrada por las proteínas IκBα, IκBβ, IκBγ, IκBδ, IκBε y Bcl-3 [295]. Las IκB contienen entre cinco y siete repeticiones conservadas de tipo ankirina, fundamentales para su unión a NF-κB a través de los dominios DHR. De este modo, el represor o la subunidad inhibitoria enmascaran la SLN e impide la

translocación al núcleo. Las proteínas IκB, poseen además una secuencia de desestabilización en la región C-terminal, que favorece su degradación proteolítica [228].

4.1. Activación de NF-κB

NF-κB se puede activar por la vía clásica/canónica, la vía alternativa/no canónica y una vía híbrida de las dos anteriores [21;98;290].

4.1.1. Activación de la vía canónica de NF-κB

En esta forma de activación los complejos NF-κB se encuentran localizados en el citoplasma celular en su estado inactivo, asociados a la proteína inhibitoria IκB. El dímero más abundante en la mayoría de los tipos celulares y por tanto el mejor caracterizado es el formado por RelA/p50. La activación clásica es generalmente una respuesta rápida y transitoria a una amplia gama de estímulos. NF-κB puede activarse por citoquinas proinflamatorias (TNFα y TWEAK), mitógenos, proteínas virales, lipopolisacárido bacteriano (LPS), factores de crecimiento, LDLox, estímulos inmunológicos y el estrés físico o químico. Tras la llegada del estímulo, se produce la activación de las quinasas IKKs (las subunidades catalíticas IKKα, IKKβ y la subunidad reguladora IKKγ o NEMO), que inducen la fosforilación de IκB (principalmente de IκBα), en dos residuos serina que marcan esta proteína para ser reconocida por el complejo proteína ligasa-ubiquitina. Esta enzima poliubiquitina a IκB, lo que facilita su degradación por el proteosoma 26S, exponiéndose la región SLN del NF-κB, así se libera el NF-κB unido a IκB. El dímero libre se transloca al núcleo, donde se une a los sitios-κB de los genes diana para activar o reprimir su transcripción (**Figura 3**)

La activación canónica de NF-κB regula la transcripción de genes que codifican múltiples proteínas pro-inflamatorias como las quimioquinas MCP-1 y RANTES o la molécula de adhesión ICAM-1.

4.1.2. Activación de la vía no canónica de NF-κB

Existe un número limitado de estímulos que activan la vía no canónica de NF-κB. Entre ellos están algunos miembros de la TNFRSF, como son CD40R, receptor de linfotoxina β, BAFFR y Fn14 [46;54;129;232;246]. La vía no canónica se caracteriza por el procesamiento de NFκB2/p100 dependiente de la activación de la quinasa IKK-α por la quinasa inductora de NF-κB (NIK) dando lugar al complejo p52/RelB. Los heterodímeros p52/RelB pueden ir directamente al núcleo [53] (**Figura 3**). Entre las dianas de NF-κB2 destacamos el factor quimiotáctico de linfocitos T, CCL21 [234].

La vía híbrida requiere la contribución de ambas vías: la vía alternativa genera el complejo y la vía clásica activa el complejo [53]. p100 puede estar también en el citosol formando heterodímeros con RelA y c-Rel. En este caso al procesarse p100 quedarían los heterodímeros p52/RelA o p52/c-Rel [191] que

pueden quedar retenidos en el citosol por las subunidades inhibitorias IκB (principalmente IκBα e IκBβ), cuya degradación es inducida a través de la activación del complejo IKK [53].

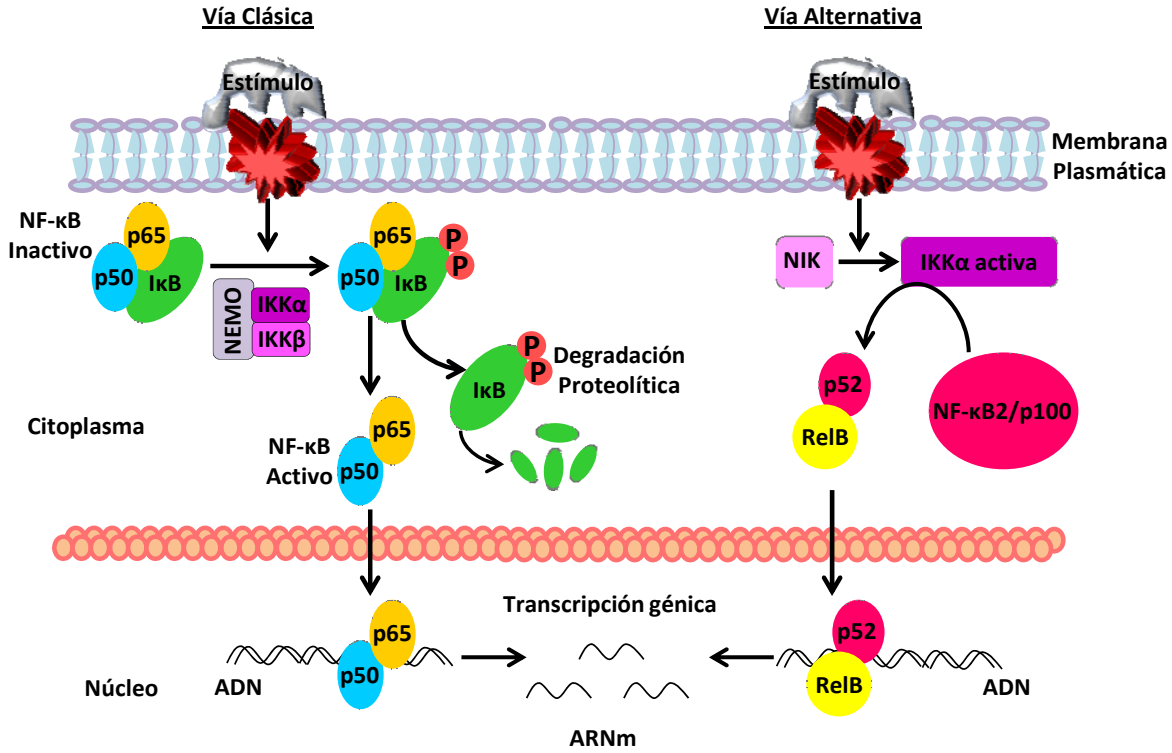

Figura 3: ***Esquema representativo de la vía clásica y alternativa de activación de NF-κB.***

A la izquierda se muestra un esquema representativo de la vía clásica de activación del factor NF-κB, y a la derecha la vía alternativa de activación.

4.1.3. Modulación de la actividad NF-κB

La regulación de la función de transactivación de NF-κB está controlada también por modificaciones post-traduccionales como la fosforilación o la acetilación de las proteínas Rel, coactivadores y corepresores de la transcripción y la acetilación de histonas, que modulan la unión al ADN con correpresores o coactivadores de la transcripción [97;98;195;290]. La acetilación/deacetilación de histonas es un paso importante en la remodelación de la cromatina que juega un papel clave en la regulación de la expresión génica. La deacetilación de histonas está mediada por las histonas deacetilasas (HDACs). Las HDACs eliminan los grupos acetilo de las histonas, incrementando así la afinidad de las histonas por el ADN. Este incremento de la unión condensa la estructura del ADN impidiendo la transcripción. De esta manera, las HDAC pueden cambiar la actividad de RelA desde la inducción a la represión de la transcripción [11;28]. La Tricostatina A y el ácido valproico son dos inhibidores de las HDACs.

4.2. Activación de NF-κB en las células renales

La activación de NF-κB ha sido estudiada en células renales intrínsecas tales como podocitos, células mesangiales, células tubulares y células endoteliales en la lesión renal o tras la exposición a estímulos inflamatorios. En las células tubulares las citoquinas de la TNFSF, como TWEAK y TNF inducen diferentes patrones de activación de genes inflamatorios [242]. Ambas citoquinas promueven la activación de la via canónica pero solo TWEAK induce la activación de la via no canónica de NF-kB, lo que sugiere que TWEAK y TNF no son citoquinas redundantes en la lesión renal [246]. NF-kB puede influir en la respuesta inflamatoria y en el daño renal por acciones adicionales a la regulación de la expresión de mediadores inflamatorios, tales como la regulación de la muerte celular y del estado de diferenciación.

4.3. NF-κB en la enfermedad renal

En la lesión renal experimental NF-κB se activa en los podocitos y en las células mesangiales durante el daño glomerular, así como en las células tubulares como consecuencia de la proteinuria o de daños tubulointersticiales primarios, incluyendo el FRA por isquemia-reperfusión, obstructivo, séptico y tóxico [86;148;230;231;242;246]. En el FRA tóxico dependiente de TWEAK se activan tanto la vía canónica como la vía no canónica de NF-κB [246]. En la enfermedad renal humana hay evidencia histológica de la activación de NF-kB en la ND, la enfermedad glomerular y el FRA. La activación de NF-κB se correlacionó con los parámetros de la gravedad de la enfermedad (tales como la proteinuria) y la inflamación [12;151;171;172;233;313].

NF-κB está implicado tanto en el inicio como en la resolución de la inflamación aguda [139;140]. Durante la resolución del daño, NF-κB podría regular a la baja genes inflamatorios, regular a la alta genes anti-inflamatorios e inducir la apoptosis de leucocitos.

Partenolide puede inhibir la actividad IKK, mejorar la estabilidad de la IκBα y/o bloquear la unión al DNA de NF-κB RelA [76;99]. La inhibición de esta ruta de señalización mediante el empleo de partenolide produce una mejora en la inflamación renal en modelos de glomerulonefritis inmune y obstrucción unilateral del uréter (UUO) [66;148]. Partenolide también disminuye la acumulación de monocitos intersticiales en el daño renal mediado por angiotensina II y en el daño renal inducido por cisplatino [71].

5. CXCL16

En los seres humanos el gen de la quimioquina CXC ligando 16 (CXCL16) está situado en el cromosoma 17p13 en un locus separado de todas las otras quimioquinas conocidas [163]. CXCL16 fue originalmente descrito como un receptor basurero de fosfatidilserina y LDLox y por ello se denominó también SR-PSOX [258]. Independientemente, CXCL16 fue identificado como un ligando para el receptor de quimioquinas CXC, CXCR6 (Bonzo, STRL33, TYMSTR) [163]. CXCL16 es una de las dos quimioquinas

ancladas a la membrana hasta ahora conocidas. La forma de membrana del CXCL16 humano es una glicoproteína transmembrana de tipo I de 254-aa y 30 kDa, que consta de un dominio quimioquina N-terminal extracelular, un tallo tipo mucina glicosilado, una región transmembrana y un pequeño dominio citoplásmico, con un motivo YXPV que es un sitio potencial de fosforilación tirosina y sitio de unión de proteína-SH2 (**Figura 3**). Las formas de CXCL16 humana y murina son también inusuales, ya que contienen seis cisteínas en el dominio quimioquina, una propiedad sólo antes observada en la subfamilia de las quimioquinas CC [15;163;213].

CXCL16 se sintetiza como un precursor intracelular que es rápidamente transportado a la superficie celular donde puede sufrir una escisión dependiente de metaloproteinasas [82]. La liberación del ectodominio está mediada por la actividad α-secretasa de dos desintegrinas y metaloproteinasas, ADAM10 y ADAM17 [253]. ADAM10 interviene tanto en la escisión constitutiva como en la inducible [2;82;115], mientras que ADAM17 parece estar involucrada sólo en la inducible [152]. La escisión de CXCL16 mediada por ADAM10 genera fragmentos C-terminal que pueden ser procesados por el complejo γ-secretasa. Esto podría generar mediadores de señalización intracelular, como se ha descrito para otras proteínas que se someten a una proteolisis intramembrana regulada [152;253] (**Figura 4**).

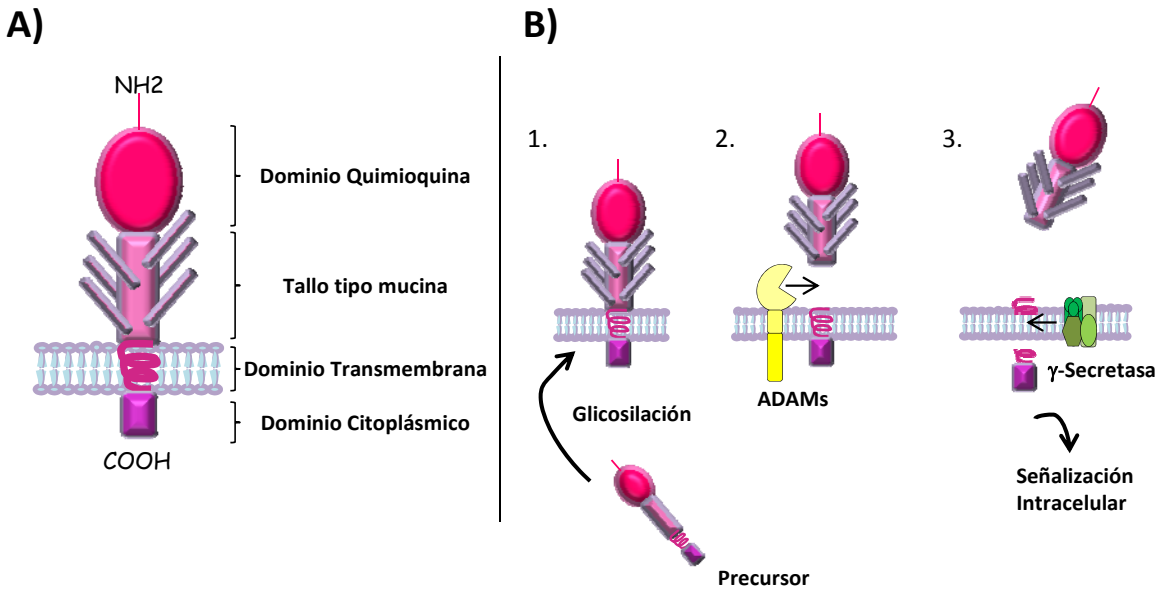

Figura 4: ***A) Estructura de CXCL16. B) Procesamiento de CXCL16. 1.*** *CXCL16 es sintetizado como un precursor intracelular que es rápidamente glicosilado y transportado a la superficie celular.* ***2.*** *El dominio quimioquina y el tallo tipo mucina son liberados por la actividad α-secretasa de ADAM10 o ADAM17.* ***3.*** *El fragmento C-terminal de CXCL16 puede ser liberado por el complejo γ-secretasa, liberando mediadores de la señalización intracelular.*

5.1. Expresión de CXCL16

Existen numerosos tipos de células que expresan CXCL16 incluyendo macrófagos, células B y T, células dendríticas, células epiteliales, hepatocitos, fibroblastos, cardiomiocitos, células musculares lisas vasculares (CMLV) y células endoteliales [48;106;163;252;257;258;296]

En el riñón humano, CXCL16 se expresa constitutivamente en las células mesangiales, los podocitos y las células tubulares, principalmente en las células tubulares distales [90;251;252] . En células glomerulares en cultivo, TNFα e IFNγ aumentan CXCL16 [90;251;252]. IFNγ aumentó la expresión de CXCL16 en cultivos primarios de células del segmento grueso ascendente del asa de Henle y en las células tubulares distales [251]. En podocitos humanos en cultivo, altas concentraciones de glucosa aumentan CXCL16 de membrana mientras que ADAM10 lo disminuye [89]. En el riñón humano normal CXCR6 se expresa en los túbulos proximales, pero no en los túbulos distales o glomérulos [91].

5.2. Funciones de CXCL16

Las funciones de CXCL16 pueden variar según el tipo celular y si CXCL16 es soluble o está unida a la membrana [118 259].

La forma unida a la membrana puede funcionar como receptor, proteína de adhesión o ligando activador del receptor CXCR6.

CXCL16 es un receptor basurero capaz de unirse y captar fosfatidilserina y LDLox [258]. CXCL16 es el principal receptor que media la captación de LDLox en los podocitos humanos [89;90]. El aumento de CXCL16 dependiente de IFNγ produce una mayor captación de LDLox por los macrófagos y pueden participar en la formación de células espumosas [304].

Unido a la membrana, CXCL16 es también una molécula de adhesión celular a las células que expresan CXCR6, promoviendo la unión y adhesión de monocitos y células T a las células endoteliales, y la adhesión de células T y NKT a células dendríticas [105;189;259]. Además en las células dendríticas CXCL16 desempeña un papel en la adhesión y fagocitosis de las bacterias [260]. CXCL16 induce eficazmente la adhesión de las células plasmáticas de la médula ósea a la fibronectina [251]. CXCL16 transmembrana en el carcinoma renal y otros cánceres suprime la proliferación del tumor [91;168].

CXCL16 soluble funciona como un quimioatrayente clásico para las células que expresan CXCR6, incluyendo las células T CD8+ y CD4+, células NK, células NKT, células plasmáticas y monocitos [105;113;131;132;189;285], y aumenta la migración transendotelial de células madre mesenquimales [263], lo que facilita la migración de células inmunes a órganos linfoides secundarios [93;95;163] y a los sitios de inflamación [20;100;132;250;287]. Además CXCL16 soluble activa una variedad de respuestas celulares en diversos tipos de células. La unión del CXCL16 soluble a CXCR6 promueve la proliferación y migración de células cancerosas in vitro e in vivo [292].

En los fibroblastos del miocardio, CXCL16 promueve la proliferación y la alteración de la síntesis de colágeno in vitro [48]. En células de musculo liso de aorta humana (HASMC), CXCL16 aumenta la adhesión célula a célula e induce la proliferación celular dependiente de NF-κB y la transcripción de genes proinflamatorios (TNFα) [33]. En células endoteliales de la vena de cordón umbilical humano (HUVEC), CXCL16 es angiogénico, estimulando la proliferación y quimiotaxis y promoviendo la formación del tubo [315].

En los podocitos renales CXCL16 promueve la quimiotaxis de células T [90], mientras que en las células mesangiales CXCL16 promueve la proliferación celular [252].

5.3. CXCL16 en el daño renal

Existen evidencias de que CXCL16 participa en la enfermedad renal. La expresión de CXCL16 se incrementa en varios modelos animales de daño renal, en la enfermedad renal humana y en fluidos biológicos de pacientes con enfermedades renales.

En modelos experimentales de nefritis lúpica murina, la expresión de CXCL16 aumenta en el suero y en la orina [274;302;303]. En la nefritis antimembrana basal glomerular (MBG) murina la expresión de CXCL16 se incrementa en los glomérulos, túbulos y el endotelio vascular, mientras que en la de rata la expresión de CXCL16 y CXCR6 aumenta en los glomérulos [75]. Tanto en la glomerulonefritis anti-MBG de rata como de ratón, los anticuerpos anti-CXCL16 fueron beneficiosos, reduciendo la proteinuria, las lesiones glomerulares y tubulointersticiales, la formación de semilunas y la infiltración de macrófagos [75;302]. En un modelo de ERC murino, inducido por UUO, CXCL16 aumenta en los túbulos y en las células intersticiales [200].

En riñones humanos de sujetos sanos, CXCL16 unido a la membrana se expresa constitutivamente en el túbulo distal, el tubulo conector y las células principales del conducto colector [251]. CXCL16 también se expresa constitutivamente en los podocitos humanos y las células endoteliales [90;303] y tiene una expresión más débil en la rama gruesa ascendente del asa de Henle [251].

En pacientes con aloinjertos renales con necrosis tubular aguda (NTA) y en pacientes con nefritis lúpica se detectan niveles elevados de CXCL16 urinarios [251;303]. En los aloinjertos renales con NTA, los niveles urinarios de CXCL16 se correlacionan con la expresión de CXCL16 en los túbulos distales y colectores [251]. CXCL16 urinario fue mayor en pacientes con NL activa que en pacientes con lupus no renal, con artritis reumatoide o en los controles normales.

CXCL16 renal aumenta en la nefropatía membranosa (NM) humana, en la ND y en los carcinomas renales. En la NM, el CXCL16 aumenta en el glomérulo. Además, el CXCL16 sérico fue mayor que en los controles, pero los valores fueron más bajos que en un paciente con nefritis lúpica [90]. En la ND, CXCL16 aumenta en los podocitos [89]. En carcinomas renales, la expresión de CXCL16 es mayor en

los de células renales papilares que en los carcinomas de células claras y los carcinomas cromófobicos [91].

6. KLOTHO

Klotho es una proteína de origen fundamentalmente renal, con propiedades antienvejecimiento, descrita como la proteína deficitaria en ratones con envejecimiento acelerado hereditario [135].

6.1. Estructura

Klotho (Kl) recibe este nombre por la diosa griega que hacía girar el hilo de la vida. Klotho fue identificado en 1997 casualmente al integrar un transgen en la región promotora del gen identificado después como Klotho [135]. Los ratones homocigotos apenas expresan Klotho y muestran múltiples trastornos, que se asemejan a los síndromes del envejecimiento humano [135].

El gen que codifica Klotho está localizado en el cromosoma 13q12 en humanos y en el cromosoma 5G3 en ratones. La región promotora del gen Klotho carece de la caja TATA y contiene cuatro sitios de unión para la proteína específica-1 (SP1) [261]. El gen humano Klotho tiene polimorfismos de nucleótido simple (SNPs) que están implicados en la aparición de patologías relacionadas con la edad. Determinados polimorfismos se asocian a una mayor expresión de Klotho [8-10;116]. En un estudio en pacientes en hemodiálisis las personas con los polimorfismos de Klotho asociados a una mayor expresión, a una mayor cantidad de la proteína, tienen mejor supervivencia que las que tienen el polimorfismo asociado a una menor expresión de la proteína [73]. Klotho posee dos transcritos, que codifican para una proteína de membrana y una proteína secretada [165;261]. El transcripto de la isoforma de membrana está formado por cinco exones y muestra la misma organización en humano y en ratón [165;261]. El transcripto de la isoforma secretada humana contiene cinco exones, mientras que el de ratón contiene tres exones. En los humanos el nivel de expresión de la isoforma secretada predomina sobre la isoforma de membrana, mientras que en los ratones es al revés [165;261].

Klotho es una proteína transmembrana de tipo I. Se compone de 1012 aminoácidos en humano y 1014 aminoácidos en ratón (135 kDa). La forma de membrana de Klotho se compone de un dominio extracelular compuesto de dos repeticiones internas (KL1 y KL2) que comparten una secuencia de aminoácidos homólogas a la β-glucosidasa [281], un dominio transmembrana y un pequeño dominio intracelular [161] (**Figura 5**). Además, contiene un pequeño grupo de aminoácidos básicos entre KL1 y KL2, que es un posible sitio de escisión proteolítica [165]. Klotho puede ser escindido por la actividad α- y β-secretasa de dos desintegrinas y metaloproteinasas, ADAM10 y ADAM17, dependientes de insulina. Estas proteinasas pueden generar dos fragmentos de Klotho de 130 kDa y 68 kDa [36]. Parte del dominio

extracelular de la proteína Klotho puede ser liberada en el espacio extracelular por escisión proteolítica y la forma secretada de Klotho puede funcionar como un factor humoral [165].

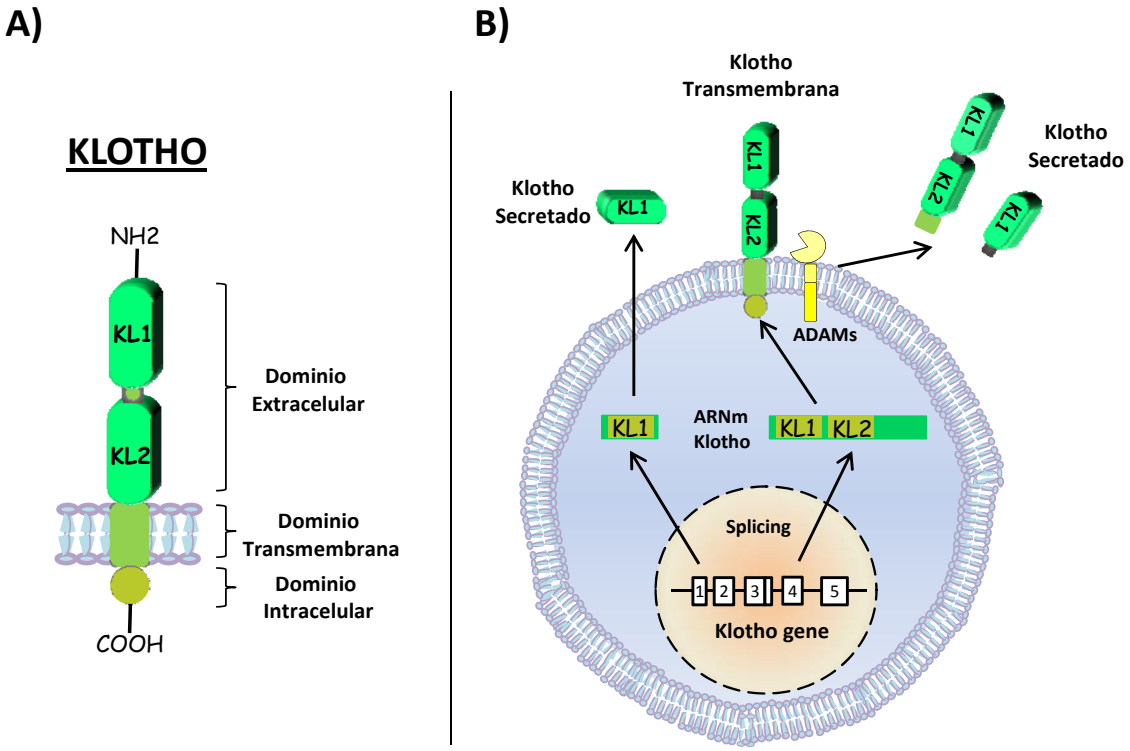

Figura 5. A) Estructura de Klotho. B) Esquema del gen, transcritos y proteínas de Klotho. *El gen klotho está compuesto por 5 exones. 2 Transcritos son generados a través de splicing alternativo de ARN. El transcripto más pequeño que contiene solo KL1, codifica la proteína de Klotho secretada, mientras que el más grande contiene las repeticiones KL1 y KL2 y codifica la forma trasmembrana de Klotho. La forma transmembrana de Klotho puede ser escindida por las ADAMs y liberada al torrente sanguíneo.*

Klotho se expresa mayoritariamente en el riñón, el cerebro (en el plexo coroideo), la glándula paratiroides, y el músculo esquelético. También se expresa en el páncreas, los ovarios, los testículos y la placenta. En el riñón, Klotho se expresa principalmente en el túbulo contorneado distal y también está presente en el plasma y la orina [135;141].

Recientemente, se ha identificado una proteína homóloga de Klotho, que se ha llamado β-Klotho, y Klotho se ha redefinido como α-Klotho [120].

6.2. Fenotipo del ratón knock out (ko) para Klotho:

El ratón ko para Klotho (Kl -/-) es un modelo animal que reproduce múltiples desordenes asociados al envejecimiento humano. Estos fenotipos son [135;197]:

- Retraso en el crecimiento y período de vida corto: Su vida media es de 2 meses (ninguno de ellos sobrevive más de 100 días), frente a los 3 años de los ratones control.
- Atrofia de los órganos genitales (testículos, útero y ovarios) y deterioro de la maduración de las células gonadales, que les impide aparearse. Las células productoras de la hormona del crecimiento, la hormona luteinizante y la hormona estimulante del folículo son más pequeñas que las de los ratones control.
- Atrofia del timo en las etapas tardías.
- La creatinina puede elevarse tardíamente hacia las 10-12 semanas.
- Calcificación ectópica (órganos y paredes arteriales) y arteriosclerosis (calcificación de la aorta, de arterias musculares de tamaño medio y de pequeñas arterias renales).
- Osteoporosis generalizada y osteopenia: Disminución de la densidad mineral ósea, del espesor del hueso cortical y del número de osteoblastos y osteoclastos.
- Alteración en el modo de andar y en el funcionamiento del sistema nervioso central (hipokinesis). Se asemeja a la manera de andar parkinsoniana del humano.
- Atrofia de la piel: Reducción en el número de folículos pilosos y en el espesor dérmico y epidérmico. La grasa subcutánea es difícilmente detectable.
- Enfísema pulmonar: Dilatación de los espacios aéreos distales a los bronquiolos terminales, acompañada de una destrucción de la arquitectura alveolar normal.
- El análisis de sangre y suero muestra hipercalcemia leve, hiperfosfatemia e hipoglucemia con disminución de la insulina en el páncreas y disminución del ratio de linfocitos a leucocitos. Los niveles proteína total, albúmina, triglicéridos y colesterol fueron normales.

Curiosamente, la mayoría de los síntomas físicos, bioquímicos y morfológicos documentados en kl-/- se relacionan con la edad indicando que Klotho suprime el envejecimiento.

6.3.Funciones de Klotho

Las funciones de Klotho pueden variar dependiendo de si se encuentra de manera soluble o está unida a la membrana. Klotho transmembrana puede funcionar como un co-receptor para el factor de crecimiento de fibroblastos (FGF)23. Además, su dominio extracelular puede ser liberado y secretado, funcionando potencialmente como un factor humoral, regulando múltiples vías de señalización y la actividad de múltiples canales iónicos. Las acciones de Klotho sobre múltiples proteínas de membrana se han relacionado con su actividad enzimática glucosidasa con la que altera los glúcidos de estas proteínas trastocando su función o su vida media. Klotho también protege a las células y los tejidos del estrés oxidativo.

6.3.1.**FGF23 y regulación de la homeostasis del fósforo**

La forma transmembrana de Klotho actúa como un co-receptor para FGF23. FGF23 es un miembro de la superfamilia de FGF que mantiene el metabolismo de la vitamina D y la homeostasis del fosfato en el riñón. Klotho forma un complejo binario con varias isoformas del receptor FGF (FGFR1c, 3c ó 4) y aumenta significativamente su afinidad a FGF23, induciendo la interacción específica entre FGF23 y FGFR [136;286]. La deficiencia de FGF23 o de Klotho genera fenotipos similares que se caracterizan por hiperfosfatemia [220], hipercalcemia [219] y elevada síntesis de vitamina D activa ($1,25(OH)_2D$) [220].

La principal función conocida de Klotho es la regulación del metabolismo del fosfato. En ratones donde el fosfato fue manipulado genéticamente o a través de la dieta sugiere que la homeostasis del fosfato aberrante es un factor clave en el síndrome de envejecimiento acelerado de los ratones Klotho -/- [197].

6.3.2.**Klotho y calcio**

En los mamíferos, la regulación de la concentración de calcio libre extracelular ($[Ca^{2+}]e$) es esencial para muchos procesos fisiológicos. Klotho regula la homeostasis del calcio a través de diferentes mecanismos:

- La actividad β-glucuronidasa de Klotho hidroliza los residuos de azúcar extracelulares del canal de calcio renal epitelial, TRPV5 (receptor transitorio miembro potencial vaniloide 5), activándolo y reteniéndolo en la membrana plasmática, lo que aumenta la reabsorción de Ca^{2+} renal [32;34;281].
- Klotho regula el sistema Na^+,K^+-ATPasa y la hormona paratoidea [119].

6.3.3.**Klotho regula múltiples vías de señalización**

Klotho soluble inhibe la señalización intracelular de la insulina y del factor de crecimiento insulínico 1 [137]. Klotho se une a multiples ligandos Wnt e inhibe su capacidad de activar la señalización de Wnt [101;144]. También activa la vía de las proteínas quinasa C (PKC) en el riñón y testículos [117]. En HUVECs, Klotho aumenta la producción de Adenosin monofosfato cíclico (AMPc) de manera tiempo y dosis dependiente [306]. También aumenta la actividad de la enzima convertidora de angiotensina I, la actividad de la manganeso superóxido dismutasa y la producción de oxido nítrico a través de la vía dependiente de AMPc-Proteina quinasa A (PKA) e inhibe la angiotensina II inducida por la producción de especies reactivas de oxígeno [221;306]. Klotho disminuye la expresión de la proteína NADPH oxidasa 2 a través de la vía dependiente de AMPc-PKA en las células de músculo liso de aorta de rata [293]. También puede regular la senescencia celular reprimiendo la vía p53/p21 en los fibroblastos humanos y HUVEC [49;159]. Y limita la señalización a través del receptor de TGFβ1 [61].

6.4. Klotho en el daño renal

La expresión de Klotho está modulada en el daño renal. Y éste, a su vez, participa en la fisiopatología de la lesión renal.

6.4.1. Regulación de la expresión de Klotho renal

Existen varios modelos animales de inflamación renal sistémica o local en los que la expresión renal de Klotho se encuentra modulada por múltiples factores. En la mayoría de los casos Klotho se encuentra disminuido. Así, la expresión renal de Klotho está disminuida en modelos de estrés inflamatorio agudo mediado por LPS [199] y los modelos de estrés circulatorio sostenido, como los de hipertensión a largo plazo, de diabetes mellitus y de insuficiencia renal crónica [4]. Klotho renal se redujo en ratones con diferentes formas de enfermedad inflamatoria intestinal y un anticuerpo neutralizante anti-TNFα atenuó la inflamación y revirtió la represión de la expresión Klotho [275]. Además, Klotho renal se reduce en modelos experimentales de daño renal crónico o agudo caracterizados por inflamación renal. En la nefropatía diabética la disminución de la expresión de Klotho renal puede ser revertida por un antioxidante, sin cambiar los niveles de glucosa en sangre [38;311]. En pacientes con diabetes y niveles de creatinina normales Klotho circulante está bajo [58]. El nivel de Klotho soluble circulante es menor en pacientes en hemodiálisis que en personas sanas [310].

Ratones con ERC inducida por uninefrectomia más daño por isquemia-reperfusión en el riñón contralateral tenían muy bajos los niveles renales, plasmáticos y urinarios de Klotho [111]. En pacientes con ERC se han comunicado resultados discordantes, que pueden depender del ensayo utilizado [58;270]. Klotho urinario desciende ya en el estadio 1 de la ERC humana [111], una etapa caracterizada habitualmente por proteinuria, pero con función renal conservada. Este concepto es interesante porque en este estadio apenas hay pérdida de células tubulares que pueda justificar una disminución de Klotho. Más bien, el cuadro histológico principal es la inflamación tubulointersticial leve como resultado de la proteinuria o de la causa de la lesión renal. Así, planteamos la hipótesis de que la inflamación podría ser la responsable del descenso de Klotho renal en los estadios tempranos de la ERC humana. En contraste con la orina, hay informes contradictorios sobre Klotho sérico en la ERC humana.

En el FRA inducido por isquemia-reperfusión en roedores, Klotho se encuentra disminuido en la sangre, la orina y los riñones, al igual que sucede en la orina de pacientes con FRA [112]. En las células tubulares renales Klotho es disminuido por citoquinas inflamatorias tales como TNFα o IFNγ [275].

6.4.2. **Participación de Klotho en el daño renal**

Existen evidencias de que Klotho es capaz de modular la patología renal. En ratas, una sola inyección de Klotho recombinante mejora el FRA incluso cuando se administra después de la lesión [112]. La transferencia génica de Klotho mediada por adenovirus mejora los niveles de creatinina sérica y el daño renal morfológico y reduce la apoptosis inducida por isquemia-reperfusión en ratas a través de HSP70 [268;271]. La sobreexpresión de Klotho puede modular el estrés oxidativo mitocondrial y mejorar el daño renal [94]. Klotho también tiene un papel como modulador anti-inflamatorio renal. En un modelo de diabetes en ratón, la disminución de Klotho contribuye a aumentar la inflamación. Tanto Klotho soluble como de membrana regulan negativamente la activación de NF-κB a través de un mecanismo que implica la fosforilación de RelA (Ser)[536] y con ello suprime la posterior producción de citoquinas proinflamatorias, como RANTES, MCP-1, IL-6 y IL-8 inducidas por TNFα [311].

II. OBJETIVOS

El fracaso renal agudo parenquimatoso carece de un tratamiento patogénico, pero se sabe que se asocia a inflamación renal. El objetivo general de esta tesis fue identificar mediante transcriptómica renal nuevos mediadores del fracaso renal agudo relacionados con la inflamación que puedan contribuir a diseñar nuevas estrategias terapéuticas. En concreto, buscamos moléculas relacionadas con TWEAK, una citoquina multifuncional que contribuye a la inflamación renal y al fracaso renal agudo in vivo. Los objetivos concretos fueron:

1. Identificar mediadores con un posible papel en la inflamación del daño renal agudo y cuya expresión durante el fracaso renal agudo experimental se correlacione con la del receptor de TWEAK, Fn14.

2. Caracterizar la regulación de la expresión de estos mediadores por TWEAK en células renales e *in vivo* y los mecanismos moleculares implicados.

3. Caracterizar la función de alguno de estos mediadores sobre células renales.

III. MATERIALES, MÉTODOS Y RESULTADOS

A fin de identificar mediadores con un posible papel en la inflamación del daño renal agudo realizamos un array transcriptómico de FRA inducido por ácido fólico. En los resultados del array buscamos ejemplos de genes relacionados con la inflamación que tuvieran una correlación positiva con la expresión de Fn14 y ejemplos de genes que tuvieran una correlación negativa con la expresión de Fn14. Entre los genes correlacionados positivamente con Fn14 elegimos la quimioquina CXCL16 para un estudio más profundo por las razones expuestas en el trabajo "TWEAK (tumor necrosis factor-like weak inducer of apoptosis) activates CXCL16 expression during renal tubulointerstitial inflammation". Y entre los correlacionados negativamente con Fn14 elegimos Klotho por su importante papel fisiopatológico y las lagunas que existen sobre su regulación.

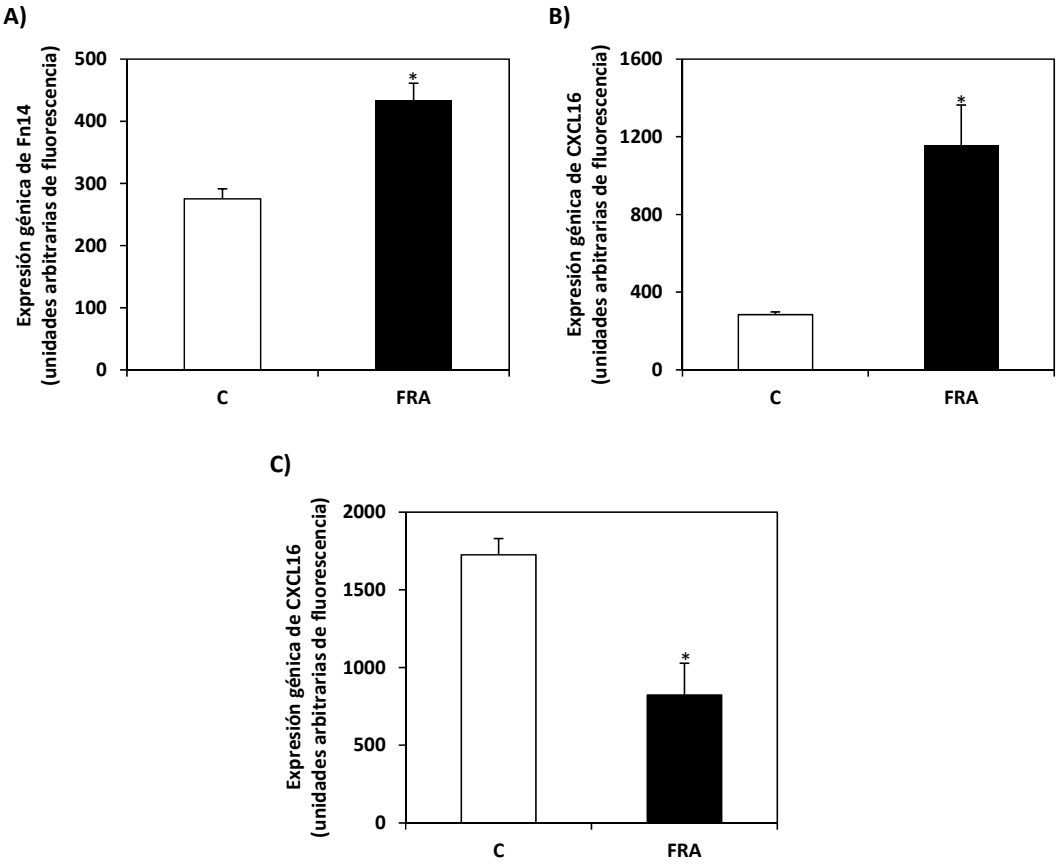

Figura 6. ***Resultados del array de transcriptómica del modelo de FRA inducido por acido fólico.*** *A) Expresión génica de Fn14. B) Expresión génica de CXCL16. C) Expresión génica de Klotho. p<0.05.*

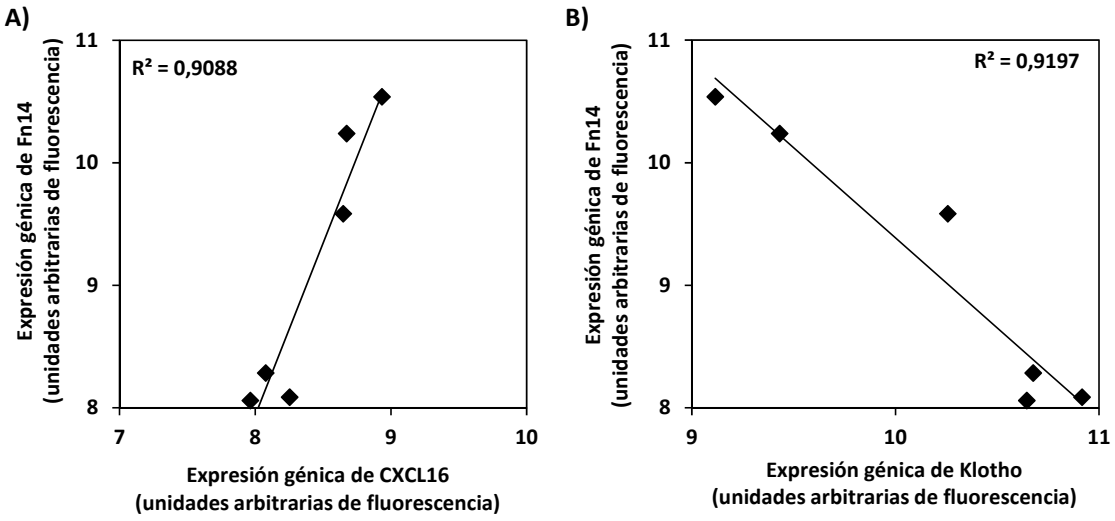

Figura 7. ***A) Correlación entre la expresión génica renal de CXCL16 y de Fn14. B) Correlación entre la expresión génica renal de Klotho y de Fn14. p<0.05.***

1. TWEAK ACTIVA LA EXPRESIÓN DE CXCL16 DURANTE LA INFLAMACIÓN TUBULOINTERSTICIAL RENAL

En el riñón, la lesión tubular desemboca en un proceso inflamatorio que intenta reparar el daño tisular, pero que a su vez, si permanece en el tiempo o es de gran intensidad, puede dañar al riñón. Así, la inflamación tubulointersticial es un componente clave en el FRA.

El objetivo general de la tesis es el conocimiento profundo y exhaustivo de las bases moleculares implicadas en el daño renal y de su progresión, para poder diseñar nuevas estrategias terapéuticas. Para buscar nuevas redes moleculares que puedan contribuir en la inflamación renal, analizamos el transcriptoma de un modelo experimental de FRA con inflamación tubulointersticial renal inducido por sobredosis de ácido fólico, donde TWEAK tiene un papel importante.

Las quimioquinas promueven la inflamación tubulointersticial renal [44]. TWEAK regula la expresión de varias quimioquinas, pero su especificidad varía en función del tipo celular [30;124;246;305]. La actividad del sistema TWEAK/Fn14 se regula fundamentalmente por los niveles de expresión del receptor Fn14. Por ello, nos centramos en buscar quimioquinas que mostraran una estrecha correlación con la expresión del receptor de TWEAK, Fn14, durante el daño renal. El análisis transcriptómico mostró un aumento significativo de la expresión de varias quimioquinas así como de Fn14 en las muestras de FRA con inflamación túbulointersticial frente a las controles. La mayoría de estas quimioquinas se liberan como mediadores solubles. Sin embargo, CX3CL1 y CXCL16 son las únicas quimioquinas conocidas hasta ahora que son sintetizadas como moléculas transmembranas y pueden ser liberadas de la superficie celular para funcionar también como quimioquinas solubles [153]. CXCL16 mostró una mejor correlación con Fn14 que la que mostró CX3CL1 con Fn14. Además CX3CL1 ha sido extensamente estudiada en el contexto del daño renal [133] en comparación con CXCL16. Por ello nos centramos en una mejor comprensión de la regulación de CXCL16 en las células renales.

In vivo TWEAK exógeno aumentó la expresión de CXCL16 y la infiltración de linfocitos T en el riñón de ratón, procesos dependientes de la vía clásica de NF-κB, ya que fueron inhibidos con partenolide. Como TWEAK moduló CXCL16 tubular *in vivo,* abordamos la relación entre TWEAK y CXCL16 en el modelo de daño tubular con inflamación túbulointersticial inducido por sobredosis de acido fólico [60;67;203;242]. Comprobamos que la expresión de CXCL16, su receptor CXCR6 y el número de linfocitos intersticiales aumentaron durante el daño, y que el anticuerpo neutralizante anti-TWEAK previno estos cambios. Los modelos animales presentan limitaciones debido a las diferencias entre especies, por lo que estudiamos que sucedía en humanos. En biopsias de riñón humano con inflamación túbulointersticial, la expresión de CXCL16 y Fn14 en las células tubulares se asoció con infiltrado inflamatorio. Tanto en el modelo murino como en las muestras humanas, CXCL16 estaba aumentado en las células tubulares del riñón. Por ello, estudiamos la relación entre TWEAK y CXCL16 en

las células tubulares renales cultivadas. TWEAK aumentó la expresión génica de CXCL16 de manera dependiente de NF-κB y la expresión proteica de CXCL16 soluble y celular.

CXCL16 renal tiene diferentes funciones dependiendo del tipo celular, pero no había datos sobre sus efectos en el túbulo renal [90;252]. Por ello, estudiamos la función de CXCL16 en las células tubulares. CXCL16 modestamente promovió la expresión de citoquinas proinflamatorias en células tubulares que expresan su receptor (CXCR6) y parece cooperar con TWEAK en promover una respuesta inflamatoria. Sin embargo, CXCL16 no moduló ni la proliferación ni la supervivencia de las células tubulares.

En resumen, TWEAK regula la expresión de la quimioquina CXCL16 en el epitelio tubular y esto puede contribuir a la inflamación túbulointersticial renal.

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TWEAK (tumor necrosis factor–like weak inducer of apoptosis) activates CXCL16 expression during renal tubulointerstitial inflammation

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TWEAK (tumor necrosis factor–like weak inducer of apoptosis) is a TNF superfamily cytokine that activates the fibroblast growth factor-inducible 14 (Fn14) receptor. Transcriptional analysis of experimental kidney tubulointerstitial inflammation showed a correlation between an upregulation of the mRNA for the transmembrane chemokine CXCL16, a T-cell chemoattractant, and Fn14 activation. Exogenous TWEAK increased mouse kidney CXCL16 expression and T-lymphocyte infiltration *in vivo*, processes inhibited by the NF-κB inhibitor parthenolide. Tubular cell CXCL16 was increased in a nephrotoxic tubulointerstitial inflammation model and neutralizing anti-TWEAK antibodies decreased this CXCL16 expression and lymphocyte infiltration. In human kidney biopsies with tubulointerstitial inflammation, tubular cell CXCL16 and Fn14 expressions were associated with inflammatory infiltrates. TWEAK upregulated CXCL16 mRNA expression in cultured renal tubular cells in an NF-κB-dependent manner and increased soluble and cellular CXCL16 protein. CXCL16 modestly promoted the expression of cytokines in tubular cells expressing its receptor (CXCR6) and appeared to synergize with TWEAK to promote an inflammatory response; however, it did not modulate tubular cell proliferation or survival. Thus, TWEAK upregulates the expression of the chemokine CXCL16 in tubular epithelium and this may contribute to kidney tubulointerstitial inflammation.

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KEYWORDS: acute kidney injury; chemokine; glomerulonephritis; inflammation

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Tubulointerstitial inflammation (TII) is a key component of both acute kidney injury and chronic kidney disease. Chemokines promote kidney TII.[1] Members of the tumor necrosis factor (TNF) superfamily of cytokines regulate several cell responses, including proliferation, differentiation, cell death, and inflammation.[2] Tumor necrosis factor–like weak inducer of apoptosis (TWEAK, TNFSF12) is a TNF superfamily member that regulates glomerular and tubulointerstitial inflammatory responses,[3–7] cell death, and cell proliferation in a cell-type- and microenvironment-dependent manner.[4,5,8–11] TWEAK signals via the fibroblast growth factor–inducible 14 (Fn14) receptor.[3,5] TWEAK regulates the expression of several chemokines, but the specific chemokines vary with cell type and are subject to differential regulation. In renal tubular cells, TWEAK promotes the canonical nuclear factor-κB (NF-κB)-dependent secretion of MCP-1/CCL2 and RANTES/CCL5, but also the non-canonical NF-κB-dependent secretion of CCL19 and CCL21.[3,12] TWEAK also upregulates MIP-1α/CCL3 and 10 kDa interferon gamma-induced protein (IP-10)/CXCL10 in mesangial cells. However, TWEAK does not upregulate MCP-1 in bronchial epithelium or keratinocytes.[12–14]

Most chemokines are released as soluble mediators.[1] However, CX3CL1 (fractalkine) and CXCL16 (SR-PSOX) are synthesized as transmembrane molecules and can be cleaved from the cell surface to produce a soluble chemoattractant.[15] Fractalkine has been extensively studied in the context of kidney disease.[16] However, much less is known about CXCL16 and kidney injury. CXCL16 was originally described as a scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein and termed SR-PSOX.[17] Independently, CXCL16 was identified as a ligand for the CXC-chemokine receptor CXCR6.[18] CXCL16 is one of two known membrane-anchored chemokines, consisting of an extracellular N-terminal chemokine domain, glycosylated mucin-like stalk, transmembrane-spanning region, and a short cytoplasmic tail.[18–20] CXCL16 potentially

functions as both a soluble chemokine and a membrane-bound adhesion molecule.[21,22] These peculiarities make a complete understanding of CXCL16 regulation in kidney cells of particular interest. Indeed, CXCL16 has been implicated in renal disease. CXCL16 expression is increased in various animal models of kidney injury and human nephropathies.[23–28] CXCL16 promotes progression of damage from acute inflammation to a progressive phase of established glomerulonephritis in rats.[26] Interestingly, elevated levels of urinary CXCL16 in patients with acute tubular necrosis[23] or lupus nephritis[27] suggest that CXCL16 may be a potential diagnostic biomarker in these kidney diseases. Despite TWEAK being another potential urinary biomarker of lupus nephritis, and a cytokine that regulates the expression of several chemokines, no previous studies had addressed the relationship between TWEAK and CXCL16. In addition, there were no prior studies on factors that regulate CXCL16 expression *in vivo* in experimental nephropathy models, and no actions of CXCL16 on renal tubular epithelium had been previously characterized.

We have now studied TWEAK regulation of CXCL16 expression *in vivo* and in tubular cells in culture, as well as CXCL16 function in these cells.

RESULTS

Transcriptomics analysis of chemokine mRNA expression in murine TII

To search for novel molecular networks that may contribute to kidney inflammation, the transcriptome of an inflammatory murine model of kidney tubular injury induced by a folic acid overdose[3,29–31] was analyzed. Several chemokines, as well as the TWEAK receptor Fn14, were upregulated (Supplementary Table S1 online). There is an extensive understanding of the role and regulation in kidney disease of many classical chemokines.[1,32] However, less is known about the transmembrane chemokines CX3CL1 and CXCL16, which were also upregulated in this model. CX3CL1 involvement in folic acid nephropathy has been previously addressed.[33] CXCL16 expression closely correlated with Fn14 expression (Supplementary Figure S1A online) and was studied in more detail.

TWEAK modulates renal CXCL16 expression and T-lymphocyte infiltration *in vivo*

Systemic TWEAK administration increases CXCL16 mRNA expression in healthy murine kidneys at 4 h (Figure 1a). This was abrogated by the NF-κB inhibitor parthenolide, indicating NF-κB dependence. *In vivo*, CXCL16 has an important role in the recruitment of T cells to sites of inflammation,[23] and, recently, TWEAK was shown to induce kidney infiltration by T cells.[34] In this regard, renal interstitial CD3-positive lymphocytes increased 4 h post TWEAK injection, and parthenolide decreased TWEAK-induced CD3-lymphocyte infiltration (Figure 1b). Kidney CD3 infiltration persisted at 24 h (not shown). Immunohistochemistry localized CXCL16 expression to tubular epithelium in control kidneys and kidneys from TWEAK-injected mice (Figure 2). Lectin binding identified the distal nephron as the main source of CXCL16 (Supplementary Figure S2 online). In addition, proximal tubular cells near glomeruli and Bowman's capsule cells near the proximal tubule expressed CXCL16 (Supplementary Figure S2 online).

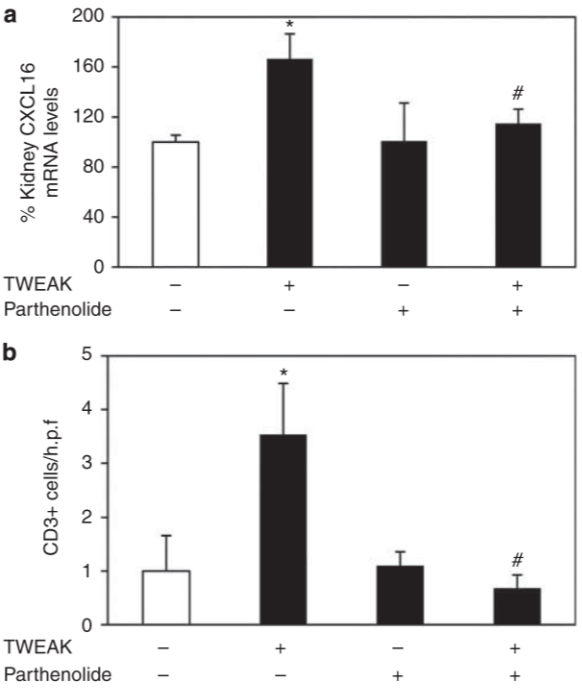

Figure 1 | TWEAK (tumor necrosis factor-like weak inducer of apoptosis) induces renal CXCL16 expression *in vivo*. (**a**) Quantification of CXCL16 mRNA by real-time quantitative reverse transcription-PCR in kidneys from mice 4 h after the administration of TWEAK and/or parthenolide. $^{*}P<0.003$ vs. control. $^{\#}P<0.05$ vs. TWEAK. Data were normalized with murine glyceraldehyde-3-phosphate dehydrogenase mRNA. (**b**) Quantification of CD3-positive cells 4 h after TWEAK or/and parthenolide injection. $^{*}P<0.009$ vs. control. $^{\#}P<0.01$ vs. TWEAK. Mean ± s.e.m. of six animals per group. h.p.f, high power field.

Neutralization of TWEAK reduces tubular CXCL16 expression in kidney TII

Acute kidney injury and progressive loss of renal function are associated with interstitial inflammation and tubular injury.[35] Tubular epithelial cells release cytokines in response to various immune and nonimmune factors contributing to attraction of inflammatory cells to the kidney.[36] As TWEAK modulates tubular CXCL16 *in vivo*, we studied in further detail the relationship between TWEAK and kidney CXCL16 expression in an inflammatory model of tubular injury induced by a folic acid overdose.[3,29–31] We had previously observed a key role for TWEAK in TII in this model, as tubular cell TWEAK and Fn14 are increased (by 3.5- and 10-fold at 72 h) and TWEAK targeting by anti-TWEAK antibodies decreased inflammation and preserved renal function.[3,37] We now report kidney CXCL16 and CXCR6

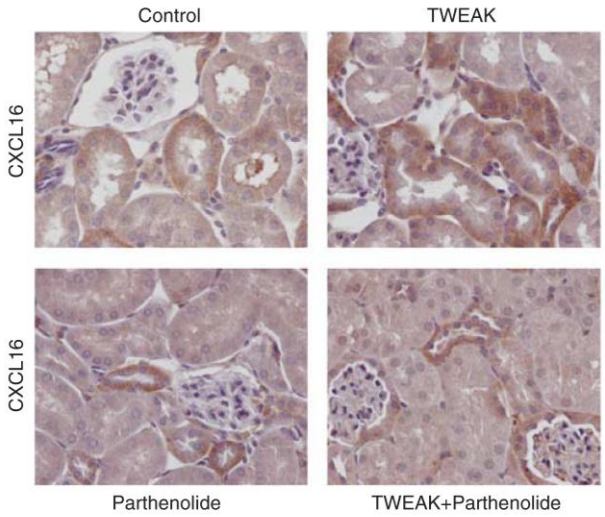

Figure 2 | Localization of TWEAK (tumor necrosis factor–like weak inducer of apoptosis)-induced renal CXCL16 expression *in vivo*. In control kidneys CXCL16 located mainly to tubules. In TWEAK-injected animals, an increased number of CXCL16-expressing tubules is noted. CXCL16 immunohistochemistry. Original magnification × 400. Pictures are representative of six animals per group.

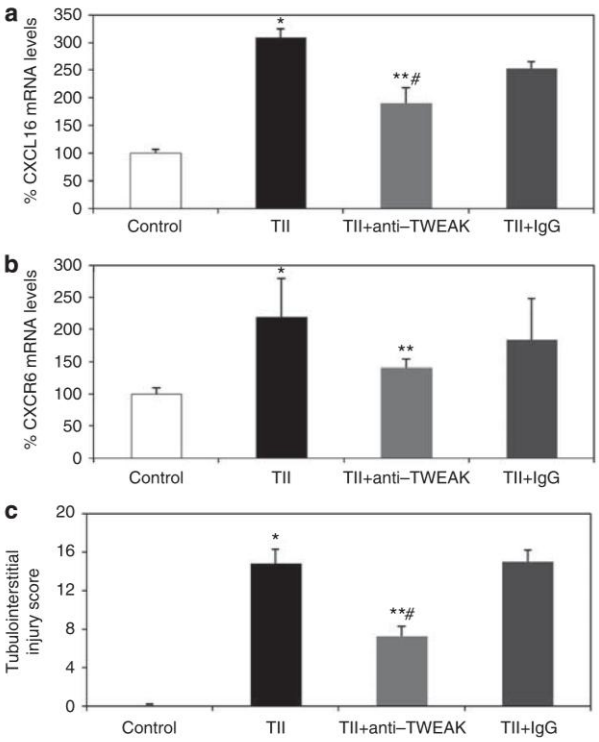

Figure 3 | TWEAK (tumor necrosis factor–like weak inducer of apoptosis) neutralization decreases CXCL16 and CXCR6 mRNA expression in experimental tubulointerstitial inflammation (TII). (**a**) During experimental TII, CXCL16 mRNA is increased. TWEAK neutralization decreased kidney CXCL16 mRNA (real-time quantitative reverse transcription-PCR (qRT-PCR)). $^{*}P<0.001$ vs. control, $^{**}P<0.01$ vs. TII, $^{\#}P<0.05$ vs. TII + IgG. Data were normalized with 18s eukaryotic ribosomal RNA. (**b**) During experimental TII, CXCR6 mRNA is increased. TWEAK neutralization decreased kidney CXCR6 mRNA (real-time qRT-PCR). $^{*}P<0.01$ vs. control, $^{**}P<0.03$ vs. TII. Data were normalized with 18s eukaryotic ribosomal RNA. (**c**) Histological assessment confirmed that cellular tubulointerstitial injury was decreased by anti-TWEAK antibodies. $^{*}P<$ 0.001 vs. control, $^{**}P<0.003$ vs. TII, $^{\#}P<0.003$ vs. TII + IgG. Mean ± s.e.m. of eight animals per group.

mRNA upregulation in this model (Figure 3). Blockade of TWEAK by anti-TWEAK antibody prevented the increased CXCL16 and CXCR6 mRNA expression, suggesting a role for TWEAK in regulating CXCL16 expression during TII (Figure 3a and b). The histological evaluation showed tubulointerstitial injury in folic acid–injected mice, which was reduced by TWEAK neutralization (Figure 3c). Immunohistochemistry localized the increased CXCL16 expression to tubular cells (Figure 4). CXCL16 expression was very prominent in the basolateral tubular membrane of some tubules and luminal side of others (Figure 4). In addition, interstitial CD3-positive lymphocytes were increased in untreated animals, and neutralization of TWEAK decreased the number of CD3 lymphocytes (Figure 5).

Increased tubular CXCL16 and Fn14 expression in human TII

CXCL16 and Fn14 protein expression levels were studied in human TII secondary to glomerular injury while minimal change disease served as a control (Figure 6). In human TII, CXCL16-positive tubules were found in close association with inflammatory infiltrates (Figure 6a). These tubules also expressed the TWEAK receptor, Fn14, and thus may be activated by TWEAK from the local environment (Figure 6b). These findings were observed in all the samples with tubulointerstitial cell infiltration. In addition, tubular expression of both Fn14 and CXCL16 (control for the technique is shown in Supplementary Figure S3 online) was observed in human acute interstitial nephritis (Figure 7).

TWEAK upregulates CXCL16 expression in renal tubular cells

Consistent with the *in vivo* data, we examined the influence of TWEAK on CXCL16 expression in cultured renal tubular cells. In proximal tubular MCT cells, CXCL16 mRNA expression was significantly increased from 3 to 24 h following exposure to 100 ng/ml TWEAK (Figure 8a). In tubular MCT cells, TWEAK activates the canonical NF-κB pathway and this is prevented by parthenolide.[3] Inhibition of NF-κB activation by parthenolide prevented TWEAK-induced CXCL16 upregulation (Figure 8b). Flow cytometry showed increased cell surface CXCL16 after TWEAK stimulation for 6–24 h (Figure 8c).

Distal tubular NP-1 cells constitutively released soluble CXCL16 into the cell culture medium (Figure 8d). TWEAK further potentiated the shedding of CXCL16 from renal tubular cells and increased cellular CXCL16 (Figure 8d). Confocal microscopy disclosed that TWEAK increased cytoplasmic and membrane CXCL16 in both MCT (Figure 9a and b) and NP-1 cells (Figure 9c). Both cell types

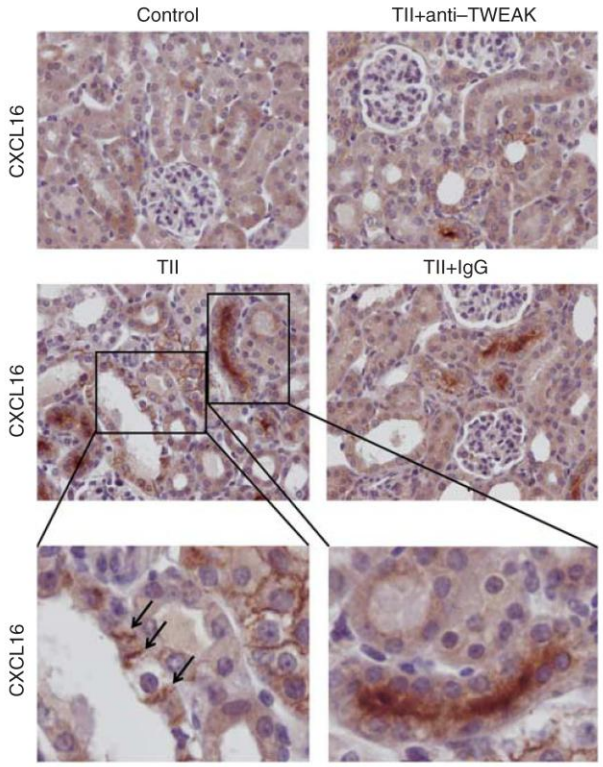

Figure 4 | Localization of CXCL16 expression in experimental tubulointerstitial inflammation (TII). Note tubular cell localization of increased CXCL16 expression in mice with TII, when compared with healthy control or mice with TII treated with anti-TWEAK (tumor necrosis factor–like weak inducer of apoptosis). Immunohistochemistry. Original magnification × 400. Pictures representative of eight animals per group.

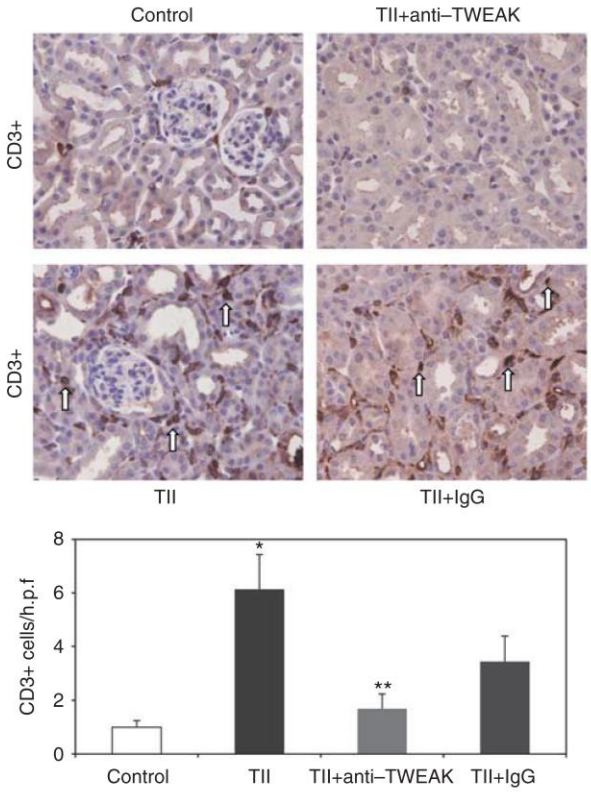

Figure 5 | TWEAK (tumor necrosis factor–like weak inducer of apoptosis) neutralization decreases interstitial CD3$^+$ lymphocytes in experimental tubulointerstitial inflammation (TII). The increased number of interstitial lymphocytes stained with anti-CD3 (arrows) in kidneys with TII was reduced by anti-TWEAK antibody treatment. Original magnification × 200. Quantification as mean ± SEM of eight animals per group. *$P < 0.01$ vs. control, **$P < 0.01$ vs. TII.

expressed the CXCL16 receptor, CXCR6 (Figure 9a–c). CXCL16 and CXCR6 were found in close association at the cell membrane in some cells (Figure 9b).

CXCL16 modulates the inflammatory response in tubular cells

TWEAK induces proliferation in nonstimulated tubular MCT cells, but the cell microenvironment modulates this response and certain inflammatory cytokines, such as TNF-α and interferon (IFN)-γ, change the response to apoptosis.[4] We confirmed that TWEAK did not promote tubular cell apoptosis but induced tubular cell proliferation[34,37] (Figure 10a and b). CXCL16 did not significantly modulate tubular cell apoptosis either alone or in combination with TWEAK (Figure 10a). CXCL16 induces proliferation in several cell types, including mesangial cells.[24,38–43] However, CXCL16 did not modulate tubular cell proliferation alone and had a nonsignificant effect on proliferation induced by TWEAK (Figure 10b). By contrast, CXCL16 had a modest pro-inflammatory effect on MCT cells and increased TWEAK-induced expression of ICAM-1, MCP-1, and RANTES mRNA (Figure 11).

DISCUSSION

The main finding of this study is that TWEAK modulates CXCL16 expression in renal tubular cells in culture and *in vivo* through activation of the NF-κB transcription factor, and that CXCL16 cooperated with TWEAK in promoting an inflammatory response in tubular cells. TWEAK neutralization decreased CXCL16 expression and CD3-lymphocyte infiltration in an experimental model of TII, suggesting the relevance of the observation for kidney injury. In this regard, tubular cell CXCL16 and Fn14 were also observed in human renal TII.

There is evidence that CXCL16 participates in renal disease. Blocking CXCL16 in the acute inflammatory phase of experimental glomerulonephritis significantly attenuated monocyte/macrophage infiltration and glomerular and tubular injury.[26,28] However, questions remain regarding the regulation of CXCL16 expression and CXCL16 actions on different intrinsic renal cells. CXCL16 is expressed by immune cells such as dendritic cells, macrophages, B cells, T cells, and on smooth muscle cells, hepatocytes, fibroblasts,

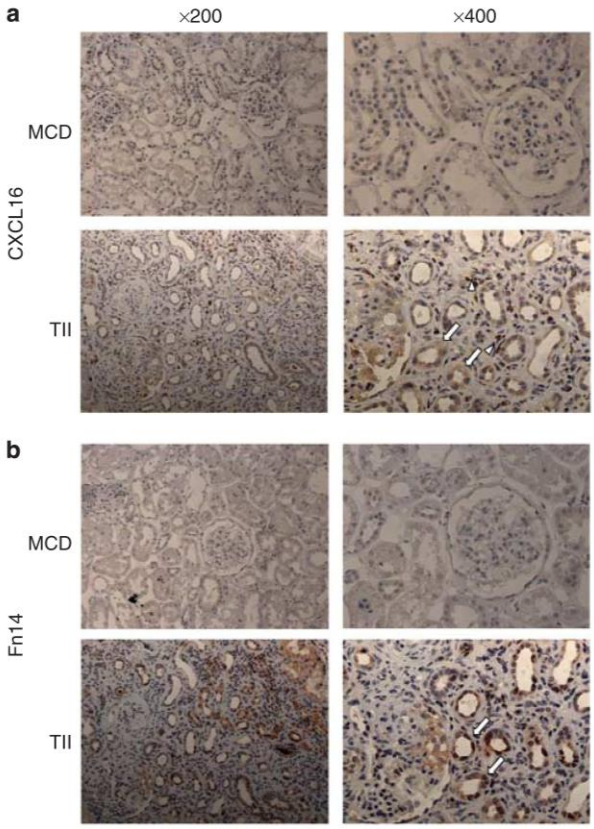

Figure 6 | Increased tubular fibroblast growth factor–inducible 14 (Fn14) and CXCL16 expression is associated with interstitial inflammatory infiltrates in human tubulointerstitial inflammation (TII). (**a**) CXCL16 immunohistochemistry. CXCL16 is observed in renal tubules (arrows) and in surrounding inflammatory infiltrates (arrowheads) in a case of TII secondary to glomerular injury (rapidly progressive glomerulonephritis), but not in minimal change disease (MCD). (**b**) Fn14 immunohistochemistry. Fn14 is observed in the same renal tubules (arrows) that were stained for CXCL16 in serial sections of the same case of TII, but not in MCD. Original magnifications × 200 and × 400. Control for the technique is shown in Supplementary Figure S3 online. Representative images of two patients with MCD, and two with rapidly progressive glomerulonephritis.

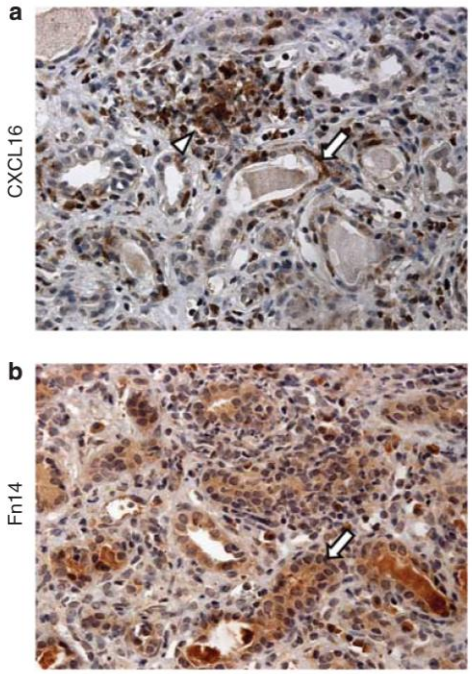

Figure 7 | Increased tubular fibroblast growth factor-inducible 14 (Fn14) and CXCL16 expression is associated with interstitial inflammatory infiltrates in human acute tubulointerstitial nephritis. (**a**) CXCL16 immunohistochemistry. CXCL16 is observed in renal tubules (arrows) and in surrounding inflammatory infiltrates (arrowheads). (**b**) Fn14 immunohistochemistry. Fn14 is observed in renal tubules (arrows) of the same case. Original magnification × 400.

and endothelial cells.[17,18,24,44–46] In addition, CXCL16 is constitutively expressed in human mesangial cells, podocytes, and tubular cells.[23–25] Glomerular CXCL16 expression is increased in human membranous nephropathy and in experimental glomerular injury.[25] In cultured glomerular cells, CXCL16 is upregulated by TNF-α and IFN-γ, but much less is known about factors regulating CXCL16 expression in renal tubular cells.[23–25] IFN-γ increased CXCL16 expression in cultured primary thick ascending limb cells and early distal tubular cells.[23] However, there is evidence for differential regulation of CXCL16 expression in different tubular segments and in tubular injury of diverse etiology. Thus, increased focally apical tubular CXCL16 expression was observed in human allograft acute kidney injury.[23] By contrast, tubular CXCL16 was mostly negative in patients with interstitial rejection and prominent interstitial T-cell infiltration.[23] The low CXCL16 expression in interstitial rejection was attributed to increase CXCL16 shedding, and remnant CXCL16 was located to the basolateral membrane. In our experimental nephrotoxic TII model, both prominent apical and basolateral CXCL16 expressions were noted. Thus, other tubular cells, the interstitium, and the tubular lumen were exposed to CXCL16 derived from tubular cells. In many tubules both patterns did not overlap.

Leukocytes recruited by chemokines have a key role in kidney tubulointerstitial tissue injury during acute kidney injury and chronic kidney disease.[47,48] CXCL16 has an essential role in T-cell recruitment,[23,25] and, recently, TWEAK was shown to induce kidney infiltration by T cells.[34] We now demonstrate that TWEAK is a novel regulator of CXCL16 expression in renal tubular epithelial cells in culture and *in vivo*. TWEAK increased renal CXCL16 expression and interstitial CD3-positive lymphocytes. TWEAK was also an important stimulus for CXCL16 expression in an experimental model of TII. In addition, neutralization of TWEAK decreased CD3-lymphocyte infiltration. Although TWEAK regulates the expression of several chemokines, we hypothesize that induction of CXCL16 expression in tubular cells may

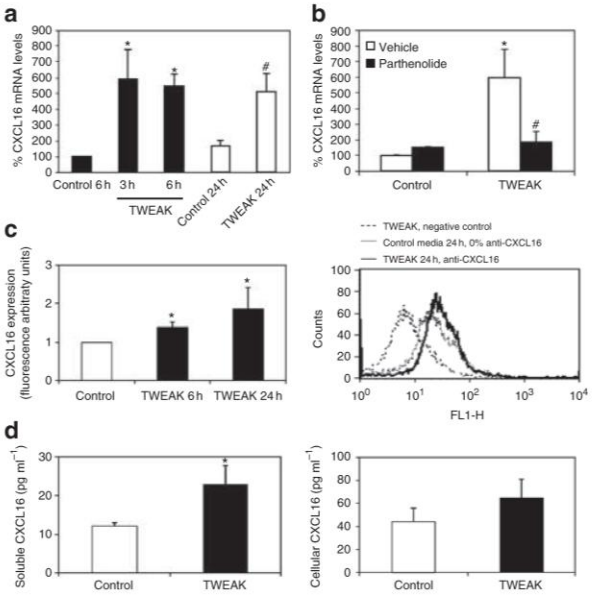

Figure 8 | TWEAK (tumor necrosis factor–like weak inducer of apoptosis) modulates CXCL16 expression in cultured renal tubular cells. (**a**) MCT cells were stimulated with 100 ng/ml TWEAK. Quantification of CXCL16 mRNA expression (real-time quantitative reverse transcription-PCR). Expression level at 6 h control was considered to be 100%. Mean ± s.e.m. of three independent experiments. $^{*}P<0.04$ vs. control 6 h; $^{\#}P<0.05$ vs. control 24 h. (**b**) MCT cells were pretreated for 1 h with 10 μmol/l parthenolide and stimulated with TWEAK for 3 h. The nuclear factor-κB inhibitor parthenolide prevented TWEAK-induced CXCL16 upregulation. Mean ± s.e.m. of three independent experiments. $^{*}P<0.05$ vs. vehicle; $^{\#}P<0.05$ vs. vehicle + TWEAK. Data were normalized with murine glyceraldehyde-3-phosphate dehydrogenase mRNA. (**c**) MCT cells stimulated with TWEAK for 6 h or 24 h were analyzed for CXCL16 surface expression by flow cytometry. Mean ± s.e.m. of three independent experiments. $^{*}P<0.03$ vs. control. (**d**) NP-1 cells were treated with 100 ng/ml TWEAK for 24 h. Soluble and cellular CXCL16 were quantified by ELISA. Mean ± s.e.m. of three independent experiments. $^{*}P<0.05$ vs. control.

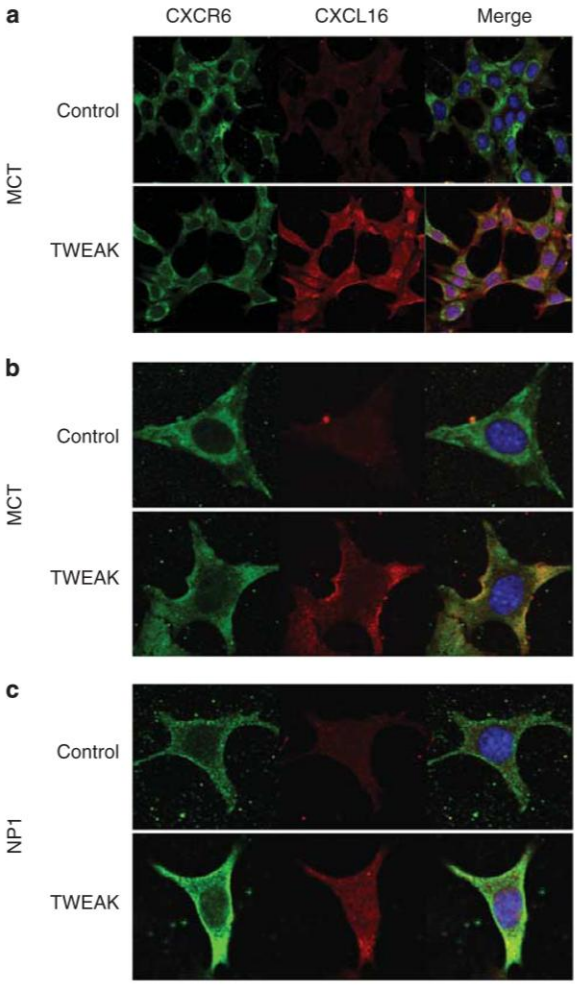

Figure 9 | Confocal microscopy detection of CXCL16 and CXCR6 expression in cultured tubular cells. Cells were stimulated with 100 ng/ml TWEAK (tumor necrosis factor–like weak inducer of apoptosis) or vehicle (control) for 24 h. (**a**) Constitutive CXCR6 (green) and inducible CXCL16 (red) expression in proximal tubular MCT cells. Original magnification × 40. (**b**) Constitutive CXCR6 and inducible CXCL16 expression in proximal tubular MCT cells. Note the increased peripheral location of CXCL16 following TWEAK with areas of overlap (yellow). Original magnification × 40, zoom 7. (**c**) Constitutive CXCR6 and inducible CXCL16 expression in distal tubular NP1 cells. Original magnification × 40, zoom 7. Indirect immunofluorescence using anti-CXCR6 with secondary Alexa Fluor 488–conjugated antibody (green) and anti-CXCL16 antibodies with secondary Alexa Fluor 633–conjugated antibody (red). Nuclei were stained with 4′-6-diamidino-2-phenylindole (blue). Images are representative of three independent experiments.

contribute to T-cell recruitment, as cell culture studies suggest, and collaborate with TWEAK in promoting inflammation. In this regard, in human interstitial inflammation, Fn14-expressing tubules also expressed CXCL16 and were surrounded by inflammatory infiltrates.

Interestingly, urinary CXCL16 excretion was increased in patients and mice with lupus nephritis, and urinary TWEAK was independently reported to be increased in these conditions.[27,49] In this regard, CXCL16 expression was increased in glomeruli and tubules in lupus mice and in experimental anti-glomerular basement membrane nephritis.[26–28] Furthermore, elevated urinary CXCL16 was observed in transplant patients with acute kidney dysfunction, in whom only tubular CXCL16 was upregulated, suggesting a tubular origin of urinary CXCL16.[23,27] TWEAK induction of CXCL16 expression in tubular cells could contribute to this observation.

CXCL16 has biological activities beyond leukocyte chemotaxis. CXCL16 function may vary in a cell-specific manner. CXCL16 induces proliferation in glial precursor cells, myocardial fibroblasts, trophoblastic cells, endothelial cells, aortic smooth muscle cells, and mesangial cells.[24,38–42] However, in cancer cells, soluble CXCL16 induces proliferation while transmembrane CXCL16 suppresses tumor proliferation.[43] Renal epithelial cell (podocyte and tubular cell)-originated CXCL16 promotes chemotaxis of T cells and may

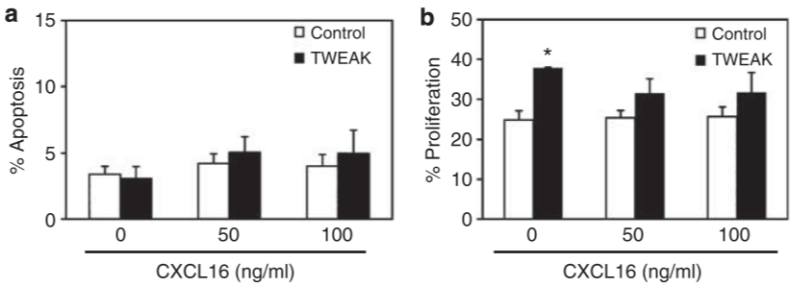

Figure 10 | CXCL16 does not modulate tubular cell survival or proliferation. (**a**) Tubular MCT cell death and (**b**) proliferation were assessed by flow cytometry of DNA content after culture for 24 h in the presence of 100 ng/ml TWEAK and different concentrations of CXCL16. Hypodiploid cells were considered apoptotic and cells in S/G2/M phase were considered proliferating. Mean ± s.e.m. of four independent experiments. $^*P < 0.02$ vs. control.

regulate the uptake of oxidized low-density lipoprotein,[25] whereas mesangial cell CXCL16 promotes mesangial cell proliferation.[24] Despite this role regulating proliferation in mesenchymal, endothelial, and tumor cells, CXCL16 did not induce murine tubular epithelial cell proliferation or apoptosis, either alone or in combination with TWEAK. TWEAK induces proliferation in non-stressed renal tubular epithelium[37] and apoptosis in cells stressed by an inflammatory milieu composed of TNF-α/IFN-γ.[4] Despite the lack of an effect of CXCL16 on tubular cell proliferation, tubular cells expressed the CXCR6 receptor and CXCL16 had a proinflammatory effect on tubular cells and increased TWEAK-induced gene expression of ICAM-1, MCP-1, and RANTES. TWEAK promotes the expression of chemokines through two distinct NF-κB pathways, the canonical and the non-canonical pathway.[12,50] The evidence presented in this paper that TWEAK promotes CXCL16 expression through the canonical (parthenolide-sensitive) NF-κB pathway extends the range of TWEAK-regulated chemokines. In this regard, TWEAK-induced chemokines may behave in a concerted manner to attract CD3 T cells and other leukocytes to the tubulointerstitial space. We cannot exclude that CXCL16 has additional actions on tubular cells, related to its role as a scavenger receptor.[17]

In summary, we have identified TWEAK as a novel regulator of CXCL16 expression in tubular cells in culture and *in vivo*, thus further extending the range of inflammatory mediators under the direct regulation of this master switch in inflammation. In addition, we have identified direct proinflammatory actions of CXCL16 in renal tubular cells. This information will be useful to define therapeutic strategies targeting TWEAK and/or CXCL16 in kidney disease.

MATERIALS AND METHODS

Cells and reagents

MCT murine proximal tubular epithelial cells were cultured in RPMI 1640, and NP-1 murine distal tubular epithelial cells were cultured in DMEM supplemented with decomplemented fetal bovine serum (10%), glutamine (2 mmol/l), and penicillin/streptomycin (100 U/ml; LONZA, Verviers, Belgium) in 5% CO_2 at 37 °C.[4,51] Both cell lines were originated from the kidneys of SJL mice in the University of Pennsylvania and obtained from Eric G Neilson and Frank Strutz, respectively.[52] Recombinant human soluble TWEAK (Millipore, Massachusetts, MA) was used at 100 ng/ml, based on prior dose–response studies in these cells.[3] Cell culture and *in vivo* experiments suggest that human and murine TWEAK have similar actions on murine renal cells.[3,34,37,53] This is not unexpected as the TWEAK and Fn14 amino-acid sequences are remarkably conserved throughout evolution, and cross-reactivity between human and mice has been documented biochemically and in other cell systems.[54] Recombinant mouse CXCL16 was obtained from R&D Systems (Minneapolis, MN). The NF-κB inhibitor parthenolide (Sigma, St Louis, MO) was used at 10 μmol/l based on previous dose–response studies in this system.[3]

Quantitative reverse transcription-PCR

Total RNA was isolated by using Trizol reagent (Invitrogen, Paisley, UK). Complementary DNA was synthesized from 1 μg of total RNA using High-Capacity complementary DNA Archive Kit, and real-time PCR reactions were performed on the ABI Prism 7500 sequence-detection PCR system (Applied Biosystems, Foster City, CA) using the DeltaDelta Ct method. Data were normalized with murine glyceraldehyde-3-phosphate dehydrogenase or 18s eukaryotic ribosomal RNA expression. Primers were designed by Applied Biosystems.

Flow cytometry analysis of CXCL16 expression

Cells were stimulated with TWEAK for 6 or 24 h. Adherent cells were detached with 2.2 mmol/l EDTA, 0.2% bovine serum albumin in phosphate-buffered saline, and resuspended in 0.2% bovine serum albumin/phosphate-buffered saline. In all, 5×10^4 cells were stained with 1.5 μg/ml anti-CXCL16 antibody overnight at 4 °C, followed by incubation with 1:100 fluorescein isothiocyanate (FITC)-anti-goat IgG for 30 min at 4 °C. Cells were then analyzed on the FACScalibur (BD Bioscience, San Jose, CA). Dead cells and debris were excluded from analysis by selective gating based on anterior and right-angle scatter. At least 10,000 events were collected for each sample, and data were displayed on a logarithmic scale of increasing green-fluorescence intensity by CELL-QUEST software.[55]

ELISA

Cells were stimulated with 100 ng/ml TWEAK for 24 h. CXCL16 was measured in cell culture supernatants and cell lysates using a DuoSet ELISA Development kit (R&D Systems), according to the manufacturer's instructions.

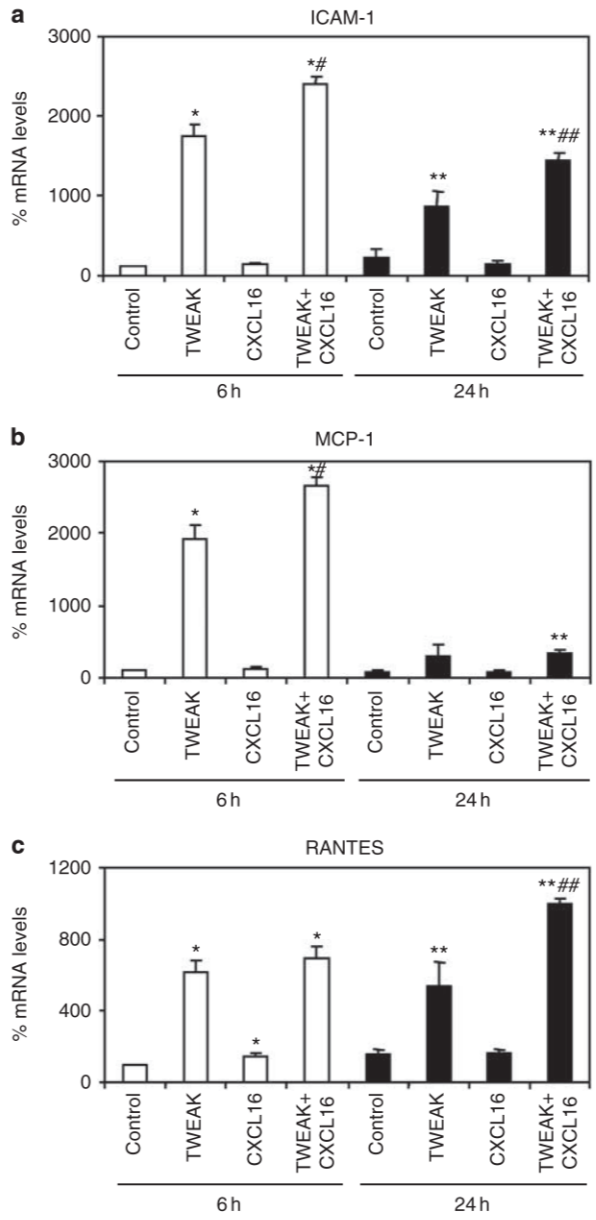

Figure 11 | CXCL16 modulates TWEAK (tumor necrosis factor–like weak inducer of apoptosis)-induced tubular inflammation. MCT cells were pretreated with 50 ng/ml CXCL16 for 1 h, and then stimulated with TWEAK for 6 h or 24 h. (**a**) ICAM-1 mRNA, $^{*}P<0.02$ vs. control 6 h, $^{**}P<0.03$ vs. control 24 h, $^{\#}P<0.05$ vs. TWEAK 6 h, $^{\#\#}P<0.05$ vs. TWEAK 24 h. (**b**) MCP-1 mRNA, $^{*}P<0.01$ vs. control 6 h, $^{**}P<0.03$ vs. control 24 h, $^{\#}P<0.05$ vs. TWEAK 6 h. (**c**) RANTES mRNA, $^{*}P<0.01$ vs. control 6 h, $^{**}P<0.03$ vs. control 24 h, $^{\#\#}P<0.05$ vs. TWEAK 24 h. Real-time quantitative reverse transcription-PCR. Values for mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Expression level at 6 h control was considered to be 100%. Mean ± s.e.m. of four independent experiments.

Immunostaining

Cells seeded in 24-well plates over glass coverslips were serum-starved for 24 h and treated with TWEAK.[25] Primary antibodies were anti-mouse CXCL16 and monoclonal anti-mouse CXCR6 (R&D Systems). Bound antibodies were detected by Alexa Fluor 633 rabbit anti-goat and Alexa Fluor 488 donkey anti-rat secondary antibodies, respectively (Invitrogen, San Diego, CA). Nuclei were counterstained with 4′-6-diamidino-2-phenylindole (Sigma, Deisenhofen, Germany). Cells were analyzed under a laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

Flow cytometry assessment of cell death

Cells were cultured to subconfluence in 12-well plates and rested in serum-free medium for 24 h before TWEAK addition. Apoptosis was assessed by functional and morphological studies.[37] Cell DNA content was quantified by flow cytometry for assessment of cell cycle and apoptosis.[56,57] Adherent cells were pooled with spontaneously detached cells, and stained in 100 µg/ml propidium iodide, 0.05% NP-40, 10 µg/ml RNAse A in phosphate-buffered saline, and incubated at 4 °C for >1 h. This assay permeabilizes the cells and thus it is not based on the known ability of propidium iodide to enter dead cells. The percentage of apoptotic cells with decreased DNA staining (A_0) and of cells in the M and S phase of the cell cycle was counted as previously described.[4,37]

Animal model

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. C57/BL6 mice (12- to 14-week-old; IFFA-CREDO, Barcelona, Spain) were treated intraperitoneally with 70 µg per mouse parthenolide or its vehicle (0.05% dimethyl sulfoxide) and 0.75 µg/mouse of TWEAK or saline vehicle (200 µl 0.9% NaCl). Treatment and control groups ($n=6$) were as follows: (a) parthenolide followed by TWEAK, (b) vehicle (dimethyl sulfoxide) followed by TWEAK, (c) parthenolide followed by saline, and (d) vehicle (dimethyl sulfoxide) followed by saline. Parthenolide was injected 24 h and TWEAK 4 h before killing. The dose of TWEAK was calculated based on *in vitro* experiments for an extracellular volume of 7.5 ml per mouse, and was previously shown to induce biological responses in kidneys.[3] The dose of parthenolide was established based on previous experience.[3]

Folic acid nephropathy is a classical model of kidney tubulointerstitial injury and inflammation.[3,29–31] C57/BL6 mice (12- to 14-week-old) received a single intraperitoneal injection of folic acid (Sigma) 300 mg/kg in 0.3 mol/l sodium bicarbonate or vehicle, and mice were killed 72 h later, at the time of peak kidney injury. Mice were dosed intraperitoneally with either 200 µg of blocking anti-TWEAK monoclonal antibody (clone P2D10, Biogen Idec) or 200 µg isotype IgG (mouseIgG2a, clone P1.17, Biogen Idec; $n=8$ per group). Animals received the monoclonal antibody treatments 1 day before the folic acid injection and 2 days thereafter. The kidneys were perfused *in situ* with cold saline before removal. One kidney was snap-frozen in liquid nitrogen for RNA and protein studies and the other fixed and paraffin embedded. Key findings were confirmed in a second, independent experiment. Kidney sections were stained with hematoxylin–eosin and Masson trichrome. Histopathological changes indicating tubular cell injury (epithelial degradation that includes apical desquamation, loss of brush border and vacuolization in cytoplasm, tubular dilatation, hyaline or cellular casts, tubulitis, mitosis, regenerative cells with increased volume of nuclei and presence of nucleoli) and interstitial inflammation were assessed by an experienced pathologist blinded to the origin of the samples.

Each of these variables was quantified semiquantitatively in a 0–3 scale to yield a global score (values 0–24) for each mouse as the sum of the individual injury scores assessed in 10 randomly chosen fields for each kidney (Supplemental Table S2 online).

Transcriptomics arrays

Transcriptomics arrays of kidney tissue were performed at Unidad Genómica Moncloa, Fundación Parque Científico de Madrid, Madrid, Spain. Affymetrix microarray analysis was performed following the manufacturer's protocol. Image files were initially obtained through Affymetrix GeneChip Command Console Software. Subsequently, Robust Multichip Analysis was performed using the Affymetrix Expression Console Software. Starting from the normalized Robust Multichip Analysis, the Significance Analysis of Microarrays was performed using the limma package (Babelomics, http://www.babelomics.org), using a false discovery rate of 5% to identify genes that were significantly differentially regulated between the analyzed groups.

Immunohistochemistry

Immunohistochemistry was carried out as previously described on paraffin-embedded 3-µm-thick tissue sections.[25] For the animal model, primary antibodies were goat anti-CXCL16 (R&D Systems) and rabbit polyclonal anti-CD3 (Dako Diagnostics, Barcelona, Spain). Dako REAL EnVision kit (Dako Diagnostics) was used for CD3 staining. Sections were counterstained with Carazzi's hematoxylin. Negative controls included incubation with a nonspecific immunoglobulin of the same isotype as the primary antibody. Quantification of CD3-stained cells was made by a blinded observer determining the total number of positive lymphocytes in 20 randomly chosen fields ($\times 40$) using the Image-Pro Plus Software (Media cybernetics, Bethesda, MD). Some sections were also incubated with the proximal tubule marker, FITC-*Tetranogolobus lotus* (diluted 1:33; Sigma), or the collecting tubule marker, FITC-*Dolichos biflorum* (diluted 1:33; Sigma), as described.[57]

Human kidney samples were obtained from 18 patients with TII secondary to glomerular injury (12 membranous nephropathy, 2 rapidly progressive glomerulonephritis, 1 systemic lupus erythematosus, 1 IgA nephropathy, 2 focal segmental glomerulosclerosis), 1 patient with acute interstitial nephritis, and 2 patients with minimal change disease controls, 10 women and 11 men, aged 43 ± 19 years, serum creatinine 1.82 ± 1.54 mg/dl, and 24 h urinary protein excretion 6.25 ± 4.61 g. Formol tissue sections (4 µm thick) were dewaxed and rehydrated with distilled water. Antigen retrieval was performed by incubating the tissue sections in sodium citrate (pH 6.0) for CXCL16 and Tris/EDTA (pH 9.0) for Fn14/TWEAK receptor. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min. The primary antibody was CXCL16 goat antibody (R&D Systems) and anti-Fn14 mouse antibody (eBiosciences, San Diego, CA) with the buffer containing 1% immunoglobulin-free bovine serum albumin overnight at 4 °C. For CXCL16, the sections were then incubated with biotinylated anti-goat antibody at 1:500 (Vector Laboratories, Burlingame, CA) for 30 min. After three rinses, they were incubated with streptavidin peroxidase (Vector Laboratories) for 30 min. The slides were incubated with hematoxylin to counterstain the sections and dehydrated and mounted with Canadian balsam (Polysciences, Warrington, PA). For Fn14, the sections were incubated with Vectastain PK 7200 detection Systems (Vector Laboratories) and the red color was developed with ImmPACT NovaRED substrate (Vector Laboratories). Negative technique controls included incubation without a primary antibody. The study protocol was approved by the local Ethics Committee and informed consent was obtained.

Statistics

Statistical analysis was performed using the SPSS 11.0 statistical software. Results are expressed as mean ± s.e.m. Significance at the $P < 0.05$ level was assessed by Student's *t*-test and nonparametric Mann–Whitney U-test for two groups and analysis of variance for three or more groups. Pearson correlation was used to assess correlation between two continuous variables.

DISCLOSURE

ABS and MCI are salaried by the FIS, AO by the Programa Intensificación Actividad Investigadora (ISCIII/Agencia Laín-Entralgo/CM), and SM by Fondecyt 1080083 (Chile). The remaining authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Chemokine mRNA upregulated in experimental kidney tubulointerstitial inflammation (TII) induced by a folic acid overdose (day 3).
Table S2. Histological scoring.
Figure S1. Correlation between kidney mRNA expression for transmembrane chemokine-encoding mRNA and Fn14 (TNFRSF12a) mRNA in the transcriptomics analysis of experimental kidney tubulointerstitial inflammation.
Figure S2. Localization of CXCL16-expressing tubular cells.
Figure S3. Negative control for the immunohistochemistry technique in human biopsies.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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Supplementary material

Supplementary Table 1. Chemokine mRNA upregulated in experimental kidney tubulointerstitial inflammation (TII) induced by a folic acid overdose (day 3). Results of a transcriptomics analysis. Chemokines with a fold-change vs control >1.5 and a FDR <0.05 are listed. Values expressed as fluorescence arbitrary units.

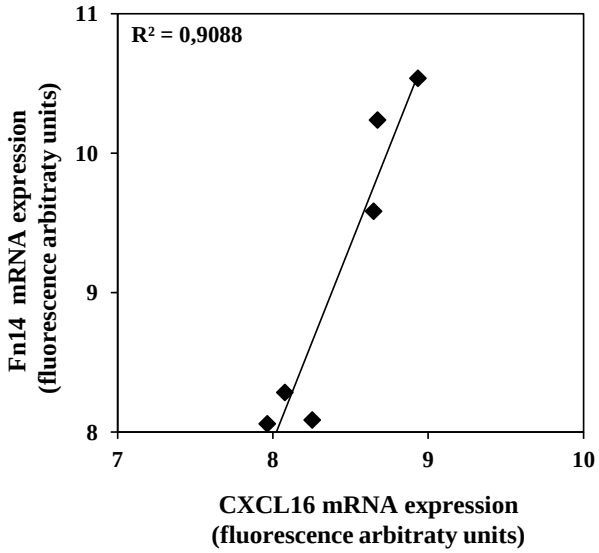
Gene Symbol	Probe Set ID	TII mean	TII DS	Control mean	Control DS	Ratio TII vs Control	adj. p-value (FDR)
Tnfrsf12a	10448307	10.12	0.49	8.14	0.12	4.07	**0.006**
Transmembrane chemokines							
Cx3cl1	10574220	10.01	0.11	9.11	0.26	1.85	**0.010**
Cxcl16	10387890	8.75	0.16	8.10	0.15	1.57	**0.014**
Classical chemokines							
Cxcl10	10531415	9.41	0.06	7.22	0.44	4.45	**0.005**
Cxcl2	10523156	5.48	0.60	3.93	0.24	3.09	**0.015**
Cxcl1	10523151	7.72	0.14	6.29	0.11	2.70	**0.003**
Ccl9	10389214	6.67	0.16	5.36	0.28	2.46	**0.006**
Ccl7	10379518	6.73	0.42	5.53	0.25	2.33	**0.015**
Ccl2	10379511	6.81	0.53	5.68	0.09	2.29	**0.022**
Ccl5	10389207	8.03	0.17	7.05	0.35	1.94	**0.015**
Ccl20	10347888	6.20	0.03	5.28	0.13	1.89	**0.005**
Ccl17	10574226	6.45	0.37	5.73	0.18	1.67	**0.045**
Cxcl14	10409579	8.77	0.30	8.07	0.14	1.64	**0.028**
Cxcl9	10531407	6.95	0.08	6.23	0.34	1.62	**0.029**
Ccl12	10379530	4.84	0.31	4.23	0.02	1.55	**0.038**
Ccl3	10389231	5.73	0.21	5.13	0.26	1.51	**0.046**

Supplementary Table 2. Histological scoring.

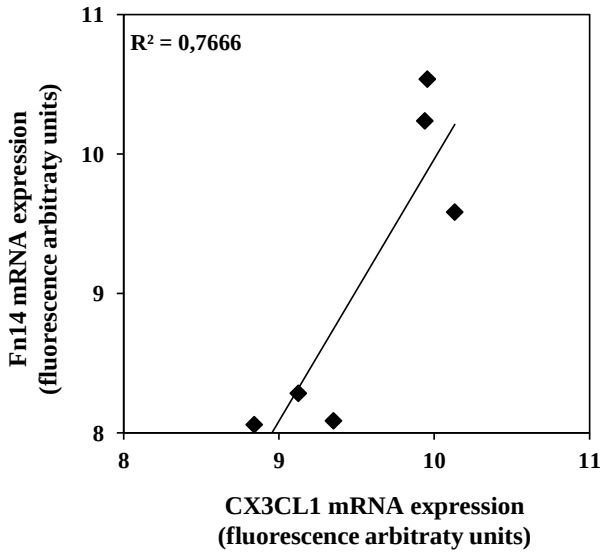
	SCORE			
	0	1	2	3
Regenerative cells with increased nuclear volume and presence of nucleoli (nuclei per x400 field)	<1	1-24	25-50	≥50
Dilated ducts: original magnification x100 (kidney section)	<10%	10-25%	>25-50%	>50%
Hyaline casts: original magnification x100 (kidney section)	<10%	10-25%	>25-50%	>50%
Cellular casts: original magnification x100 (kidney section)	<10%	10-25%	>25-50%	>50%
Mitosis: original magnification x200 (kidney section)	0	1-2	3-5	>5
Epithelial degeneration: original magnification x200 (kidney section)	<10%	10-25%	>25-50%	>50%
Tubulitis: original magnification x400 (cells/tubular section)	<1	1-2	3	>3
Inflammation: original magnification x200 (foci/ kidney section)	<1	1-2	3	>3

Supplementary Figure 1

A)



B)

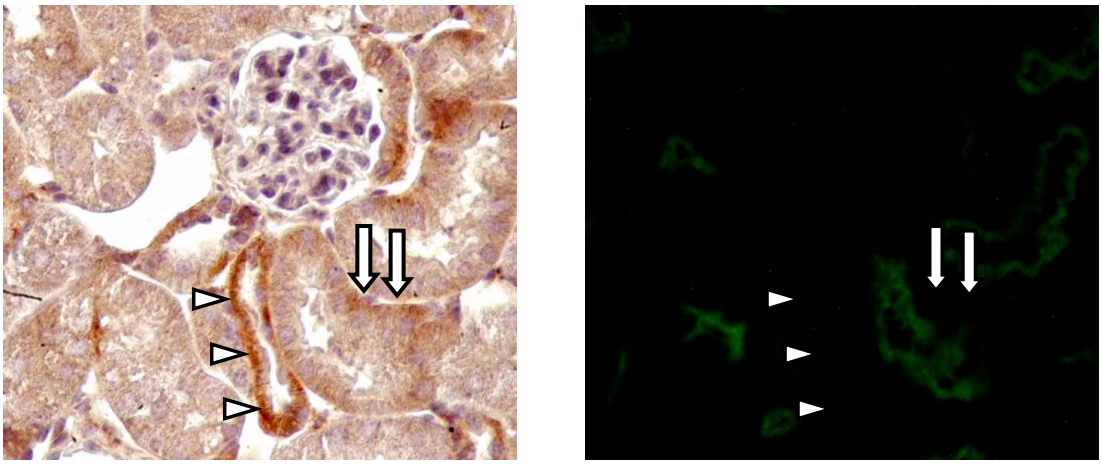


Supplementary figure 1. Correlation between kidney mRNA expression for transmembrane chemokine-encoding mRNA and Fn14 (TNFRSF12a) mRNA in the transcriptomics analysis of experimental kidney tubulointerstitial inflammation. A) CXCL16 mRNA. p<0.003 B) CX3CL1 mRNA. p<0.005.

Supplementary Figure 2

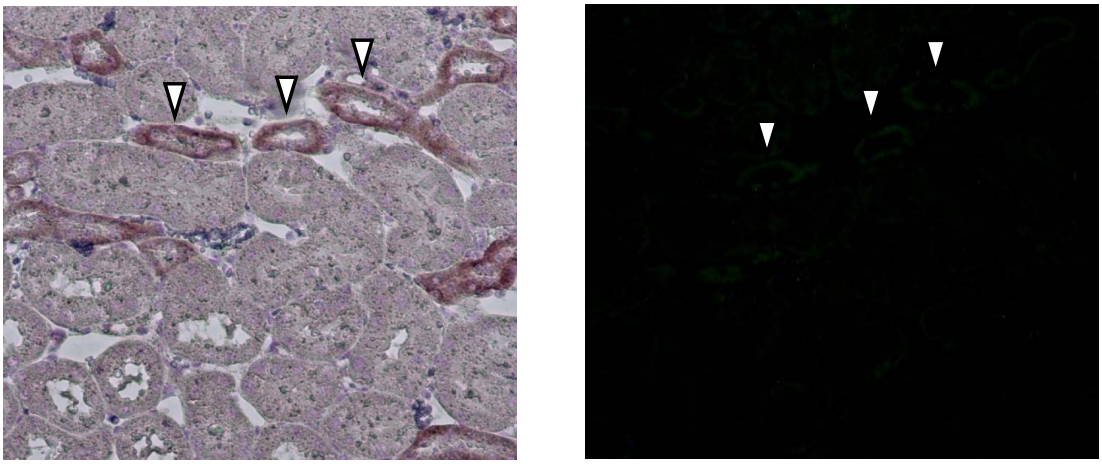
A)

CXCL16 **PROXIMAL TUBULE MARKER**



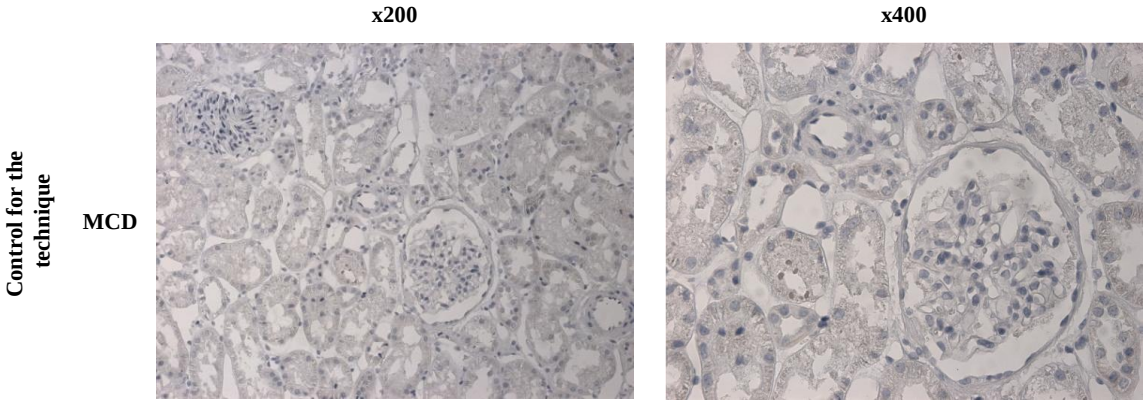
B)

CXCL16 **DISTAL TUBULE MARKER**



Supplementary figure 2. Localization of CXCL16-expressing tubular cells. A) CXCL16-expressing tubular cells were localized in TWEAK-injected mice by staining with proximal tubule marker FITC-Tetranogolobus lotus. Arrows point to CXCL16 positive proximal tubules and nearby Bowman´s capsule cells with a proximal tubular lectin staining pattern. Arrowheads identify intensely stained non-proximal tubules. **B)** By contrast, in control mice distal nephron segments identified by FITC-*Dolichos biflorum* staining are the main CXCL16-expressing tubular cells. Arrowheads identify intensely stained distal nephron tubules.

Supplementary Figure 3



Supplementary figure 3. Negative control for the immunohistochemistry technique in human biopsies

2. LAS CITOQUINAS INFLAMATORIAS TWEAK Y TNFα REDUCEN LA EXPRESIÓN RENAL DE KLOTHO A TRAVÉS DE NF-κB

Como hemos comentado anteriormente, la inflamación tiene un papel importante en el FRA. El daño renal y la inflamación se asocian con características del envejecimiento acelerado, tales como el daño vascular y la calcificación. El daño renal, el daño vascular y la calcificación son tres de los múltiples trastornos que aparecen en el envejecimiento de los ratones knockout para Klotho [197]. Klotho es una proteína expresada por las células renales que tiene propiedades antienvejecimiento, antioxidantes y antiinflamatorias [135;137]. Estudios recientes han revelado que la expresión de Klotho disminuye en condiciones patológicas que dañan al riñón y que la disminución de la expresión de Klotho a su vez exacerba el daño renal [134]. Por lo tanto la interrupción de este círculo vicioso podría proteger a los riñones del daño. Por ello estudiamos la relación entre la inflamación y la expresión de Klotho, caracterizando su regulación en las células renales por dos citoquinas proinflamatorias que participan en el daño renal, TWEAK y TNFα [126;205;242].

El análisis del array transcriptómico del modelo experimental de FRA reveló una disminución significativa de la expresión de Klotho. Además, la expresión de Klotho estaba inversamente correlacionada con la expresión de Fn14. En paralelo a nuestros estudios se describió que la inflamación sistémica consecuencia de una enfermedad inflamatoria intestinal disminuía la expresión renal de Klotho y que el antagonismo de TNF prevenía este efecto [275]. En el contexto de la lesión renal aguda inducida por ácido fólico, el bloqueo o la ausencia de TWEAK previno la disminución de los niveles plasmáticos y renales de Klotho. Este podría ser un efecto directo de TWEAK o una consecuencia indirecta de la mejora del tejido dañado [242]. Así, exploramos si TWEAK regulaba directamente la expresión renal de Klotho *in vivo*. La administración exógena de TWEAK en ratones disminuyó la expresión de Klotho en el riñón. TWEAK podría haber reclutado mediadores secundarios del daño *in vivo* que condujeran a la disminución de la expresión renal de Klotho. Por ello, estudiamos si TWEAK regulaba directamente la expresión de Klotho *in vitro* y utilizamos TNFα como control positivo [275]. Tanto TWEAK como TNFα disminuyeron la expresión de Klotho en las células tubulares. Como en las células epiteliales tubulares murinas TWEAK y TNFα activan NF-κB, exploramos si la disminución de Klotho era mediada por NF-κB. La proteína inhibitoria IκBα se une a RelA y la mantiene inactiva en el citoplasma. Así, el bloqueo con un siRNA de IκBα facilitaría la translocación nuclear de RelA y la activación de NF-κB. Tanto TWEAK, TNFα como el siRNA de IκBα activaron NF-κB y disminuyeron la expresión de Klotho. Mientras que la inhibición de NF-κB con partenolide impidió la disminución de Klotho inducida por TWEAK o TNFα. Además, TWEAK promovió la unión de RelA al promotor de Klotho, induciendo su deacetilación.

La remodelación de la cromatina por medio de la acetilación/deacetilación de histonas juega un papel importante en la regulación de la expresión génica. La deacetilación de histonas mediada por las HDACs puede cambiar la actividad de NF-κB RelA desde la inducción a la represión transcripcional [11;28]. La inhibición de las HDACs por medio de la tricostatina A o del ácido valproico evitó la disminución de Klotho inducida por TWEAK o por TNFα. TWEAK y TNFα estimularon la asociación de RelA con HDAC1 en el núcleo. Además, TWEAK indujo la deacetilación de las histonas H3 y H4 en el promotor de Klotho.

En conclusión, las citoquinas inflamatorias, como TWEAK y TNFα, reducen la expresión de Klotho a través de un mecanismo dependiente de NF-κB. Estos resultados pueden explicar parcialmente la relación entre la inflamación y las enfermedades que se caracterizan por un envejecimiento acelerado.

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The Inflammatory Cytokines TWEAK and TNFα Reduce Renal Klotho Expression through NFκB

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ABSTRACT

Proinflammatory cytokines contribute to renal injury, but the downstream effectors within kidney cells are not well understood. One candidate effector is Klotho, a protein expressed by renal cells that has antiaging properties; Klotho-deficient mice have an accelerated aging-like phenotype, including vascular injury and renal injury. Whether proinflammatory cytokines, such as TNF and TNF-like weak inducer of apoptosis (TWEAK), modulate Klotho is unknown. In mice, exogenous administration of TWEAK decreased expression of Klotho in the kidney. In the setting of acute kidney injury induced by folic acid, the blockade or absence of TWEAK abrogated the injury-related decrease in renal and plasma Klotho levels. TWEAK, TNFα, and siRNA-mediated knockdown of IκBα all activated NFκB and reduced Klotho expression in the MCT tubular cell line. Furthermore, inhibition of NFκB with parthenolide prevented TWEAK- or TNFα-induced downregulation of Klotho. Inhibition of histone deacetylase reversed TWEAK-induced downregulation of Klotho, and chromatin immunoprecipitation showed that TWEAK promotes RelA binding to the Klotho promoter, inducing its deacetylation. In conclusion, inflammatory cytokines, such as TWEAK and TNFα, downregulate Klotho expression through an NFκB-dependent mechanism. These results may partially explain the relationship between inflammation and diseases characterized by accelerated aging of organs, including CKD.

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Acute kidney injury (AKI) and progressive loss of renal function are associated with interstitial inflammation and tubular injury.[1] Members of the TNF superfamily of cytokines are key mediators of renal injury, and the transcription factor nuclear factor-kappaB (NFκB) is a mediator of their biologic activity.[2–4] TNF has pleiotropic actions on glomerular and tubular cells that contribute to renal damage.[5,6] More recently, TNF-like weak inducer of apoptosis (TWEAK, TNFSF12) was identified as a mediator of kidney tubulointerstitial inflammation through interaction with its receptor Fn14.[7,8] During kidney injury TWEAK may have additional actions, not shared with TNF, such as noncanonical activation of NFκB resulting in expression of CCL21.[9] Both TWEAK and TNF may promote tubular cell injury and death.[8,10] Cell death

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contributes to tubular cell loss in both AKI and chronic kidney disease (CKD).[11] In addition, TNF and TWEAK are mediators of inflammation and atherosclerosis, which are key events in CKD patients exhibiting high mortality rates.[12–14] In fact, CKD has features consistent with accelerated aging such as accelerated atherosclerosis. α-Klotho or Klotho is a kidney-secreted hormone with antiaging properties.[15,16] Klotho is a pleiotropic protein with glucuronidase activity that modulates calcium transport by TRPV5,[1] serves as a necessary coreceptor for fibroblast growth factor 23 (FGF23),[17] regulating Pi homeostasis,[18] inhibits Wnt signaling,[19] has antioxidant properties,[20] and represses IGF-1 signaling.[16]

Klotho is downregulated during kidney injury. Kidney Klotho mRNA is reduced in long-term hypertension, diabetes mellitus, and CKD models[21] as well as in experimental ischemia-reperfusion-induced AKI,[22] and in uremic hyperplastic parathyroid glands.[23] Decreased expression of Klotho mRNA and protein was confirmed in humans with CKD.[24] Because renal failure and inflammation are both associated with accelerated aging features, such as vascular injury and calcification, that are also observed in Klotho knock-out (KO) mice, we examined the relationship between inflammation and Klotho expression.

The factors regulating Klotho expression are poorly understood. LPS administration decreased Klotho mRNA, suggesting that the expression of Klotho is modulated by acute inflammatory stress *in vivo*.[25] However, the specific molecules that downregulated Klotho could not be identified in cell culture experiments. Angiotensin II downregulates Klotho mRNA expression in tubular cells both in culture and *in vivo*, but the intracellular molecular mechanisms were not addressed in detail.[26,27] Oxidative stress also downregulates Klotho.[28,29] Statins and Rho kinase inhibitors prevented angiotensin-induced downregulation of Klotho in cultured cells.[27] Antioxidants, statins, and Rho kinase inhibitors are known to prevent activation of NFκB in response to stimuli such as angiotensin II.[30–32]

Despite evidence relating inflammation to low Klotho expression and to accelerated aging, there had been a poor understanding of the inflammatory mediators that downregulate Klotho and of the intracellular mechanisms involved. A role for NFκB had not been characterized. We now report that TWEAK and TNF promote the NFκB-dependent downregulation of Klotho expression in cultured tubular cells and in the kidney *in vivo*.

RESULTS

Klotho mRNA and Protein Are Downregulated in Acute Inflammatory Tubular Injury

Folic acid nephropathy is a model of AKI characterized by acute renal failure and tubulointerstitial inflammation.[7] In this AKI model there is an increased renal expression of the inflammatory cytokines belonging to the TNF superfamily—TWEAK and TNFα—and of their receptors: Fn14 for TWEAK and TNFR1 and

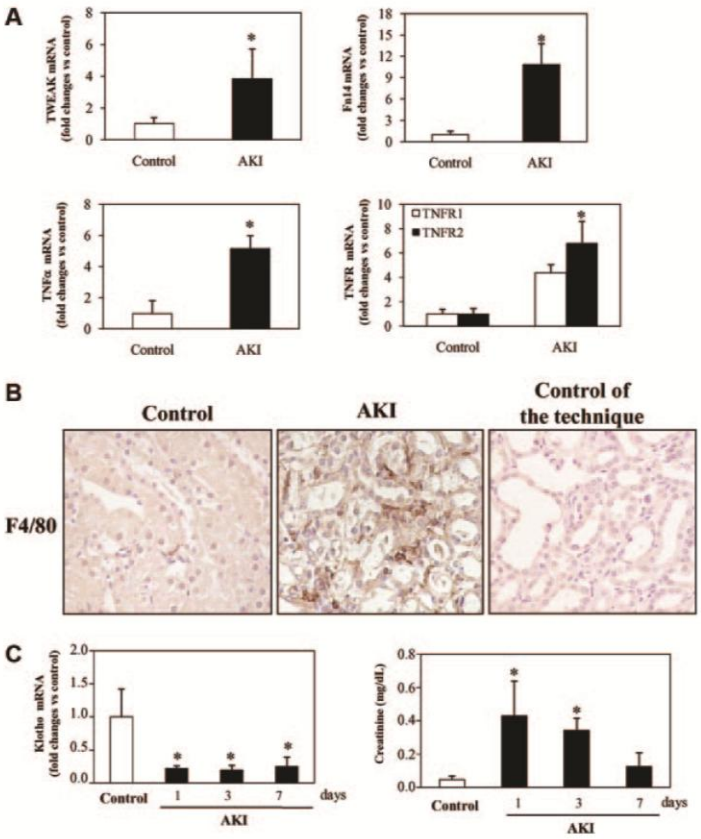

Figure 1. Cytokine and Klotho expression in murine model of renal tubulointerstitial inflammation. (A) Expression of inflammatory cytokines (TNFα and TWEAK) and TWEAK and TNF receptors (Fn14 and TNFR1/TNFR2, respectively) mRNA were measured by real time RT-PCR in kidneys of mice with AKI induced by a folic acid overdose at 72 hours. Expression of cytokines and their receptors is increased in this model. Mean ± SEM of six animals per group. *$P < 0.05$ *versus* control. (B) Representative immunohistochemistry of renal macrophage infiltration at 72 hour AKI mice Macrophages were identified by staining with F4/80 antibody. Increased macrophage-positive cells were noted in AKI mice with respect to healthy control mice. Controls for the technique are stained with nonspecific IgG. (C) Time course of kidney Klotho mRNA expression and plasma creatinine levels in folic acid-induced AKI. Kidney Klotho mRNA is decreased even when renal function has recovered. Mean ± SEM of six animals per group. *$P < 0.005$ *versus* control.

TNFR2 for TNFα (Figure 1A).[8,33,34] This is associated with tubulointerstitial inflammation characterized by a five-fold ($P < 0.002$ *versus* control) increased number of interstitial F4/80-positive macrophages (Figure 1B) and by activation of the inflammation-related transcription factor NFκB in tubular cells.[7,35] Klotho mRNA expression was decreased in experimental AKI induced by folic acid (Figure 1C) or cisplatin (Supplemental Figure S1). The time course was studied in folic acid-induced AKI. Interestingly, Klotho downregulation remained significant after recovery of renal function (Figure 1C).

Treatment with neutralizing anti-TWEAK antibody prevented the decrease in Klotho mRNA and protein expression in folic acid-induced AKI, suggesting a contribution of this cytokine to the downregulation of renal Klotho expression (Figure 2, A and C). Absence of TWEAK in TWEAK KO mice also prevented the reduction in renal Klotho mRNA during AKI, compared with wild-type (WT) mice (Figure 2B). However, the basal mRNA expression of Klotho in untreated WT or TWEAK KO kidney was similar. Moreover, Klotho plasma concentration was also reduced in folic acid-induced AKI, and treatment with anti-TWEAK neutralizing antibodies prevented the decrease in Klotho plasma levels (Figure 2D). We confirmed that the decreased renal function during AKI was significantly improved by neutralizing anti-TWEAK antibody pretreatment (Figure 3, A and B). Moreover, in TWEAK KO mice with AKI, renal function and histologic AKI score were preserved compared with WT mice with AKI (Supplemental Figure S2).

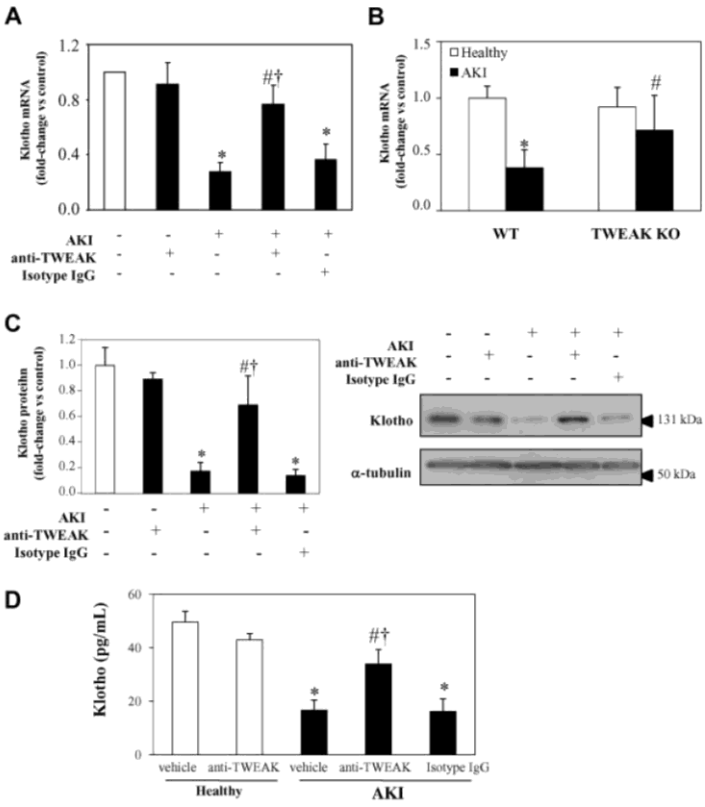

Figure 2. Antagonism or absence of TWEAK prevented downregulation of Klotho in renal tubulointerstitial inflammation. (A and B) The decreased total kidney Klotho mRNA expression observed in folic acid-induced AKI at 72 hours (RT-PCR) is improved by (A) TWEAK antagonism, using a neutralizing anti-TWEAK antibody (mean ± SEM of six animals per group; $^{*}P < 0.002$ *versus* healthy mice; $^{\#}P < 0.02$ *versus* AKI; $^{\dagger}P < 0.02$ *versus* AKI+Isotype IgG) or by (B) TWEAK absence as shown by comparing WT and TWEAK KO mice (mean ± SEM of five animals per group; $^{*}P < 0.005$ *versus* WT healthy; $^{\#}P < 0.03$ *versus* WT AKI). (C) Total kidney Klotho protein, measured by Western blot, is also reduced in AKI at 72 h, and this reduction is prevented by TWEAK antagonism. Mean ± SEM of six animals per group. $^{*}P < 0.002$ *versus* healthy mice; $^{\#}P < 0.02$ *versus* AKI; $^{\dagger}P < 0.02$ *versus* AKI+Isotype IgG. (D) Plasma levels of Klotho, measured by ELISA, are decreased in mice with AKI at 72 hours, and this is improved by anti-TWEAK antibody pretreatment. Mean ± SEM of six animals per group. $^{*}P < 0.005$ *versus* healthy mice; $^{\#}P < 0.05$ *versus* AKI; $^{\dagger}P < 0.05$ *versus* AKI+Isotype IgG.

In Vivo Exogenous TWEAK Decreases Renal Klotho through NFκB Activation

The beneficial effect of TWEAK blockade in preventing downregulation of renal Klotho expression in AKI might be a direct effect or an indirect consequence of ameliorated tissue injury.[7] Thus, we explored whether TWEAK directly regulated kidney Klotho expression *in vivo*. Systemic injection of TWEAK decreased renal Klotho mRNA levels *in vivo* at 4 and 24 h (Figure 4A). We have previously shown that the kidney proinflammatory action of TWEAK depends on NFκB activation in tubular cells.[7] In this regard, Klotho downregulation induced by TWEAK was prevented by treatment with the NFκB inhibitor parthenolide (PTN) (Figure 4A), suggesting that reduction of Klotho expression by TWEAK is mediated by NFκB activation. We confirmed by Western blot that TWEAK reduced expression of Klotho at the protein level at 24 h (Figure 4B).

TWEAK and TNFα Decreases Klotho Expression in Cultured Renal Tubular Cells

TWEAK administration may have recruited secondary mediators of injury *in vivo* that lead to the decreased renal Klotho expression. Therefore, we explored whether TWEAK and TNFα, an inflammatory cytokine from the same family that also activates NFκB, directly regulate Klotho expression in cultured tubular cells. We have previously shown that both, TWEAK and TNFα, increase the expression of a variety of genes implicated in inflammation through the activation of NFκB.[7] Thus, TWEAK and TNF increased the mRNA expres-

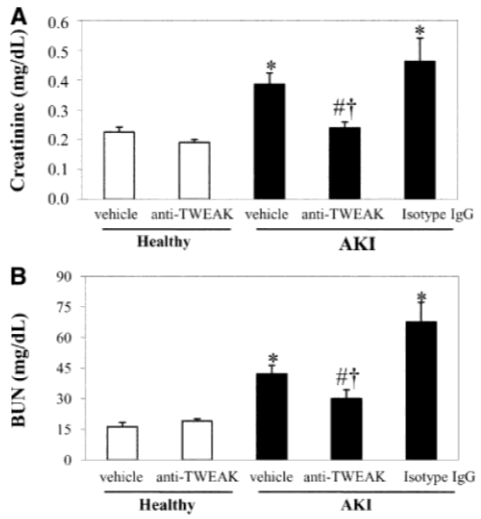

Figure 3. TWEAK neutralization improves renal function during AKI. AKI was induced by a folic acid overdose in mice and renal function was assessed at 72 hours measuring plasma creatinine and BUN. (A) Plasma creatinine levels. (B) BUN. Mean ± SEM of six animals per group. $^{*}P < 0.004$ *versus* healthy mice; $^{\#}P < 0.04$ *versus* AKI; $^{\dagger}P < 0.03$ *versus* AKI+Isotype IgG.

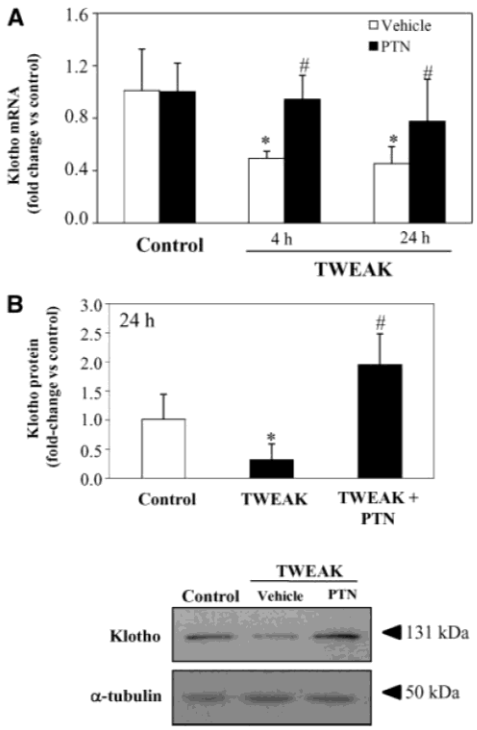

Figure 4. Systemic TWEAK injection in healthy mice decreases renal Klotho mRNA and protein *in vivo* in healthy mice. TWEAK or vehicle were injected intraperitoneally. Total kidney Klotho mRNA and protein were decreased in a time-dependent manner in TWEAK-injected mice, compared with vehicle-treated control mice. This effect was reverted by pretreatment with Parthenolide (PTN). (A) Quantitative RT-PCR analyses of renal Klotho mRNA in mice 4 or 24 hours after TWEAK, PTN, or TWEAK/PTN. Mean ± SEM of six animals per group. $^{*}P < 0.01$ *versus* control; $^{\#}P < 0.05$ *versus* TWEAK alone. (B) Total kidney Klotho protein levels in mice treated with TWEAK and PTN for 24 hours measured by Western blot. Mean ± SEM of six animals per group. $^{*}P < 0.02$ *versus* control; $^{\#}P < 0.02$ *versus* TWEAK alone.

sion of bona fide NFκB RelA/p65 target inflammatory genes such as MCP-1 and IL-6 (48- and 28-fold for TWEAK and 26- and 12-fold for TNF at 3 h in renal tubular cells, $P < 0.05$).[7] Here we show that both TWEAK and TNFα decreased Klotho mRNA expression in a dose-dependent manner by 3 h (Figure 5, A through C). Moreover, TWEAK and TNF also decreased Klotho protein (Figure 5D). Finally, Klotho concentration in cell supernatants also decreased after TWEAK treatment (Figure 5E).

TWEAK blockade with an anti-TWEAK antibody prevented Klotho downregulation induced by TWEAK, confirming the specificity of the interaction (Figure 6A). Anti-TNF neutralizing antibodies prevented TNF-induced Klotho mRNA downregulation (data not shown). TWEAK required binding to Fn14 because the anti-Fn14 neutralizing antibody ITEM-4 prevented TWEAK-induced Klotho mRNA downregulation (Figure 6B).

NFκB Mediates TWEAK- and TNFα-induced Klotho Downregulation

In murine tubular epithelial cells TWEAK and TNFα increased the DNA binding activity of NFκB as assessed by electrophoretic mobility shift assay (EMSA) (Figure 7A) and induced the translocation of the RelA/p65 subunit from the cytoplasm to the nucleus (Figure 7B). Degradation of IκBα is required for RelA translocation to the nucleus. In tubular cells, Klotho mRNA expression was decreased in cells transfected with a siRNA targeting IκBα, indicating that Klotho expression is regulated by NFκB (Supplemental Figure S3). PTN inhibits NFκB activation by preventing the degradation of IκBα.[36] PTN prevented RelA translocation and NFκB activation induced by either TWEAK or TNFα (Figure 7B). Although most known actions of NFκB RelA involve induction of gene transcription, it may also actively repress gene expression.[37] In this regard, we observed that PTN prevented the downregulation of Klotho mRNA expression induced by TWEAK or by TNF (Figure 7C). Moreover, chromatin immunoprecipitation (ChIP) assays showed RelA binding at the murine Klotho promoter in murine proximal tubular epithelial (MCT) cells upon TWEAK stimulation for 60 minutes (Figure 8A and B). These results suggest that the inflammatory cytokines, TWEAK and TNFα, decreased Klotho expression through NFκB activation.

Histone Deacetylase Activity Is Required for TWEAK-/TNFα-decreased Klotho Expression

Histone acetylation/deacetylation is an important step in chromatin remodeling that plays a key role in regulation of gene

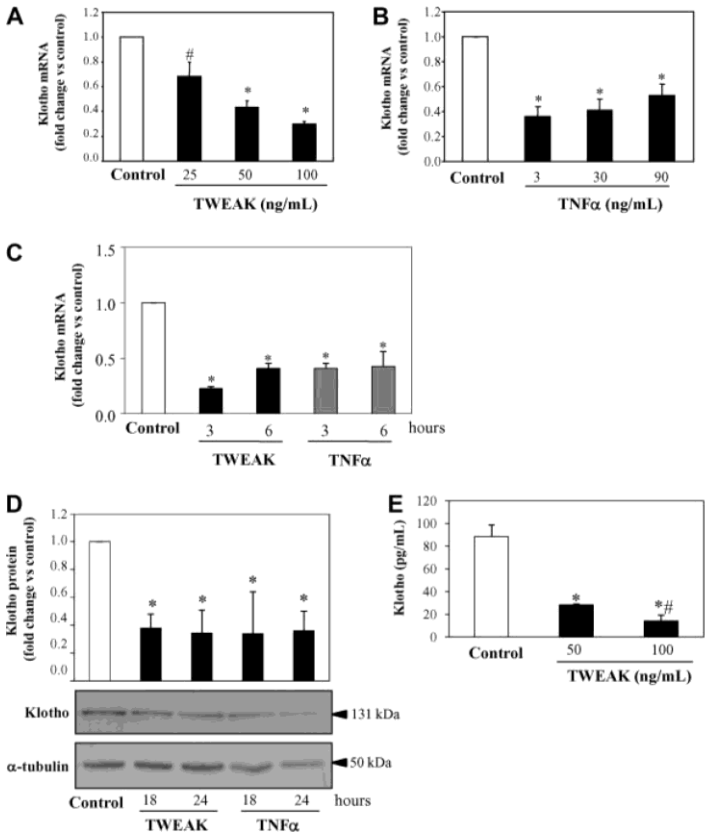

Figure 5. Inflammatory cytokines decrease Klotho expression in cultured tubular cells in an NFκB-dependent manner. TWEAK (A) and TNFα (B) decrease Klotho mRNA expression in a dose-dependent manner at 3 hours in MCT cells. Mean ± SD of three independent experiments. $^{*}P < 0.0001$ *versus* control; $^{\#}P < 0.001$ *versus* control. (C) Time course of TWEAK (100 ng/ml) and TNFα (30 ng/ml) decreased Klotho mRNA expression in tubular cells. Mean ± SD of three independent experiments. $^{*}P < 0.0001$ *versus* control. Real time RT-PCR. (D) Representative Western blot and quantification of three independent experiments shows decreased Klotho protein in MCT cells treated with TWEAK (100 ng/ml) or TNFα (30 ng/ml). Mean ± SD of three independent experiments. $^{*}P < 0.008$ *versus* control. (E) MCT cells were treated with 100 or 50 ng/ml TWEAK for 24 hours, and Klotho levels were measured in cell supernatants by ELISA. TWEAK decreased soluble Klotho in a dose-dependent manner. Mean ± SD of three experiments. $^{*}P < 0.05$ *versus* control; $^{\#}P < 0.05$ *versus* TWEAK 50 ng/ml.

expression. Histone deacetylation mediated by histone deacetylase (HDAC) generally leads to transcriptional repression and may change RelA activity from induction to repression of transcription.[37,38] Thus, we tested the modulation of TWEAK-/TNFα-induced downregulation of Klotho expression by the HDAC inhibitors trichostatin A (TSA) or valproic acid. TSA or valproic acid pretreatment prevented Klotho downregulation induced by TWEAK or TNFα (Figure 9A and Supplemental Figure S4, respectively). Moreover, RelA is associated with HDAC1 in nuclei of TWEAK- or TNFα-stimulated cells as assessed by immunoprecipitation (Figure 9B). ChIP assays using antibodies against acetylated histones corroborated that TWEAK promoted histone H3 and H4 deacetylation at the murine Klotho promoter (Figure 9C).

DISCUSSION

The main finding of our study is that in kidney cells Klotho is negatively regulated by inflammation, and it is downregulated by an NFκB RelA-dependent mechanism. We have identified individual inflammatory cytokines such as TWEAK or TNFα that downregulate Klotho in cell culture and *in vivo*. Both proinflammatory cytokines signal through NFκB RelA to increase the expression of a variety of inflammatory mediators.[7,39] However, in the case of Klotho, NFκB RelA appears to downregulate gene expression in an inflammatory milieu. These findings may have therapeutic implications in kidney injury and inflammation-associated premature aging.

The Klotho gene encodes a single-pass transmembrane protein that binds to multiple FGF receptors and functions as a co-receptor for FGF23, a bone-derived hormone that suppresses phosphate reabsorption and vitamin D biosynthesis in the kidney. In addition, the extracellular domain of Klotho protein is shed and secreted, potentially functioning as a humoral factor. The secreted Klotho protein can regulate multiple growth factor signaling pathways, including insulin/IGF-1 and Wnt, and the activity of multiple ion channels. Klotho also protects cells and tissues from oxidative stress, yet the precise mechanism underlying this activity remains to be determined. As a result, lack of Klotho in mice results in accelerated aging.[40] The kidney is the main site of Klotho expression, and kidney injury, as corroborated by our findings, results in reduced renal Klotho expression.[25,41] We may speculate that a decreased renal expression of Klotho may have local and systemic adverse consequences. Regarding potential systemic effects, reduced kidney Klotho expression during AKI persisted beyond recovery of renal function and was associated with decreased circulating Klotho. Decreased renal, circulating, and urine Klotho was recently reported in ischemia-reperfusion-induced AKI.[42] Patients with kidney disease have progressive injury to multiple organs, among them the cardiovascular system, resulting in a mortality rate which is ten times that of age-matched controls, resulting in effective accelerated

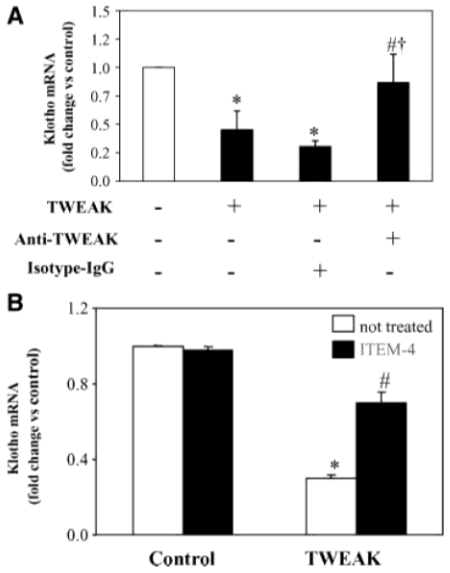

Figure 6. Fn14 mediates TWEAK-induced Klotho downregulation in cultured tubular cells. (A) A neutralizing anti-TWEAK antibody (10 μg/ml) rescued Klotho levels in MCT cells treated with 100 ng/ml TWEAK for 3 hours. Mean ± SD of three independent experiments. *$P < 0.002$ *versus* control; #$P < 0.02$ *versus* TWEAK alone; †$P < 0.006$ *versus* TWEAK+Isotype IgG. (B) ITEM-4 (10 μg/ml), a neutralizing anti-Fn14 antibody, prevented Klotho downregulation induced by TWEAK in MCT cells, suggesting that this effect of TWEAK is mediated through Fn14 binding. Mean ± SD of three independent experiments. *$P <$ 0.005 *versus* control; #$P < 0.02$ *versus* TWEAK alone.

aging.[14,43] Interestingly, systemic inflammation also promotes cardiovascular morbidity and mortality and has been associated with other age-dependent diseases such as osteoporosis and dementia.[44,45] Locally, a reduction of Klotho expression contributed to progression of kidney failure; although its overexpression ameliorated renal injury in a mouse model.[46] Ad-Klotho gene transfer improved serum creatinine and reduced renal apoptosis induced by ischemia-reperfusion in rats.[22] Klotho transfection reduced H_2O_2-induced tubular cell apoptosis in culture.[29] Klotho is also expressed in extrarenal cells. Thus, Klotho expression is reduced in human CD4 lymphocytes from aged patients.[47]

Our results show that TWEAK and TNFα induce Klotho downregulation in a dose-dependent fashion in cultured renal cells, that TWEAK decreases kidney Klotho *in vivo*, and that targeting TWEAK prevents Klotho downregulation during kidney injury. Evidence suggesting that RelA activation is necessary for TWEAK- and TNFα- induced Klotho repression includes the following: (*1*) IκBα downregulation by siRNA, a maneuver that allows RelA translocation to nuclei, promoted Klotho downregulation, (*2*) an inhibitor of RelA activation, PTN, prevented TWEAK- and TNFα-induced Klotho downregulation, (*3*) the temporal pattern of Klotho repression observed is consistent with that of RelA activation induced by TWEAK and TNFα, (*4*), and RelA is bound to Klotho promoter in MCT cells stimulated with TWEAK.[7,36] NFκB-mediated downregulation of gene expression had previously been observed for TNFα, such as downregulation of argininosuccinate synthase, endothelial nitric oxide synthase, and Bmp4 gene expression.[48–50] However, it had not been observed for TWEAK. TWEAK activates at 24 h a subset of NFκB2 protein complexes that are different from those observed after TNFα stimulation.[9,51] However, both cytokines share the early activation of RelA-containing NFκB complexes.[7,9,51] The fact that both TWEAK and TNFα downregulate Klotho and that this is an early event argues against an involvement of NFκB2, which is only activated by TWEAK and whose activation takes place after changes in Klotho mRNA have already occurred.

The main "switch" in NFκB activation is cytoplasmic and leads to the nuclear accumulation of NFκB proteins. In addition, recruitment of NFκB to chromatin is regulated in a promoter-specific manner. This kinetic complexity in NFκB-dependent gene induction is dependent on simultaneously activated pathways and transcription factors.[52] In addition to gene-specific factors there are stimulus-specific factors that determine the activator or repressor activity of NFκB on gene transcription.[37] Thus, NFκB induced by noncytokine cytotoxic stimuli is functionally distinct from NFκB induced by TNFα: the first is an active repressor of antiapoptotic gene expression, whereas the latter promotes antiapoptotic gene expression.[37] There are different mechanisms for p65/RelA-dependent gene repression. Recently, it has become apparent the importance of HDAC activity. HDAC activity is required for p65/RelA-dependent repression of PPARδ in human keratinocytes, of antiapoptotic genes in fibroblasts, and of PDGF in smooth muscle cells.[37,53,54] We observed that TWEAK and TNFα increased nuclear RelA association with nuclear HDAC1 at the same time that Klotho expression was decreased in tubular cells. This result suggested that TWEAK- and TNFα-induced Klotho downregulation could be mediated by HDAC activity. In this regard, we observed that HDAC inhibitors, TSA and valproic acid, prevented repression of Klotho induced by TWEAK or TNFα. Furthermore, TWEAK induces histone H3 and H4 deacetylation at the murine Klotho promoter in renal tubular cells.

Although TWEAK targeting by either neutralizing antibodies or in TWEAK KO mice preserved kidney Klotho expression and circulating Klotho levels and also preserved renal function, our studies do not allow us to conclude that Klotho preservation has a role in renal function improvement. However we can speculate on a nephroprotective role of Klotho based on the beneficial effect of Klotho overexpression on progressive renal injury[55] and in AKI.[42,56]

In summary, the inflammatory cytokines TWEAK and TNFα downregulate Klotho in renal tubular cells through an NFκB-dependent mechanism. These results are of interest to kidney injury and to the accelerated aging observed in uremic patients. They identify an additional adverse consequence of NFκB activation in kidney injury. In this regard, our results may be relevant to design therapeutic approaches to regulate Klotho expression.

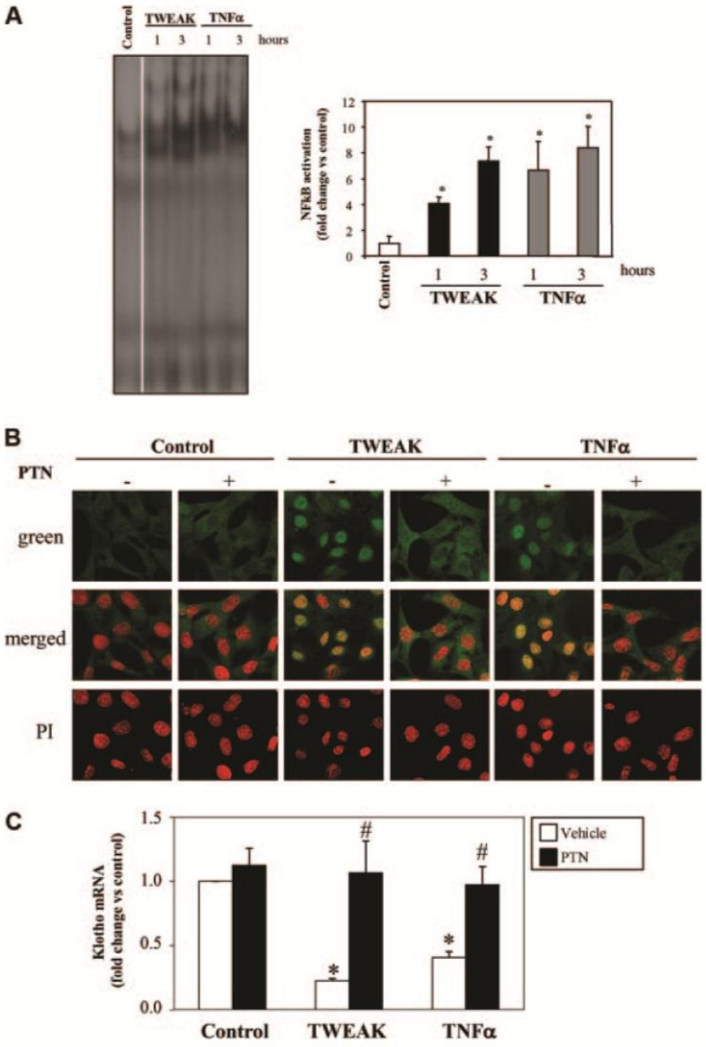

Figure 7. PTN prevents Klotho downregulation induced by TNFα or TWEAK in cultured tubular cells. (A) TNFα or TWEAK promoted DNA binding of NFκB complexes as assessed by EMSA. Quantification (mean ± SD) and representative EMSA of three independent experiments. $^{*}P < 0.05$ *versus* control. (B) TNFα or TWEAK promote the translocation of the RelA/p65 protein to the nucleus at 60 minutes in tubular cells. This is prevented by PTN. Nuclear translocation of NFκB subunits is required for DNA binding. Images representative of three independent experiments. Confocal microscopy where RelA is green and propidium iodide (nuclei) is orange (magnification, ×320). (C) The NFκB inhibitor PTN prevents TWEAK- and TNFα-induced downregulation of Klotho mRNA in cultured MCT cells at 3 hours. Mean ± SD of three independent experiments. $^{*}P < 0.0001$ *versus* control; $^{\#}P < 0.0001$ *versus* cytokine alone.

CONCISE METHODS

Cells and Reagents

MCTs were cultured in RPMI 1640, 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, in 5% CO_2 at 37°C.[7]

RPMI-1640, penicillin, streptomycin, and trypsin-EDTA were from BioWhittaker (Waltham, Massachusetts), FBS from Life Technologies (Carlsbad, California), and recombinant human TWEAK from Chemicon, Millipore (Billerica, Massachusetts). Anti-TWEAK monoclonal antibody (clone P2D10) and specific isotype control (clone P1.17) (Biogen Idec) were used at 10 μg/ml. ITEM-4, neutralizing anti-Fn14 antibody (eBioscience, San Diego, California) was used at 10 μg/ml. Anti-TNF polyclonal antibody was used at 3 μg/ml (BioLegend, San Diego, California). PTN (Sigma, St. Louis, Missouri) at 10 μM inhibits NFκB activation in MCT cells without decreasing cell viability.[7] HDAC inhibitors, TSA (Upstate Biotechnology, Millipore) and valproic acid (Sigma), were used at 100 ng/ml and 3 mM, respectively. Klotho concentration in cell supernatants and mice plasma was determined with the E97757Mu ELISA Kit (USCNK, Wuhan, P.R.China).

Animal Models

Studies were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. C57/BL6 mice (12 to 14 weeks old) (IFFA-CREDO, Barcelona, Spain) were treated with 70 μg/mouse PTN or its vehicle (0.05% DMSO) and 0.75 μg/mouse of TWEAK or saline. Treatment and control groups ($n = 6$) were as follows: 1) PTN followed by TWEAK, 2) vehicle (DMSO) followed by TWEAK, 3) PTN followed by saline, and 4) vehicle (DMSO) followed by saline. PTN was injected 24 h before that TWEAK injection and TWEAK 4 or 24 h before sacrifice. The dose of TWEAK was calculated based on *in vitro* experiments for an extracellular volume of 7.5 ml/mouse. The dose of PTN was established based on previous experience.[7]

Folic acid nephropathy is a classical model of AKI.[7,57–59] C57/BL6 mice (12 to 14 weeks old) received a single intraperitoneally injection of folic acid (Sigma) 250 mg/kg in 0.3 M sodium bicarbonate or vehicle, and mice were killed 24 or 72 h or 7 d later.[7] Mice were dosed intraperitoneally with either 200 μg neutralizing anti-TWEAK mAb (clone P2D10, Biogen Idec)[7] or 200 μg isotype IgG (mouse IgG2a, clone P1.17, Biogen Idec) ($n = 6$ per group). In another set of experiment, TWEAK KO (Biogen Idec)[60] mice on the C57Bl/6 background strain received intraperitoneally folic acid injection, and they were killed 72 h later ($n = 5$ per group).

Cisplatin is a nephrotoxic routinely used in clinical in cancer treatment. C57/BL6 mice (12 to 14 weeks old) received a single intraperitoneally injection of cisplatin (Sigma, 20 mg/kg in saline) or vehicle, and mice were killed 72 h later ($n = 5$ per group). The dose was derived from preliminary studies in our mice colony. Mean plasma creatinine in cisplatin AKI mice was 0.8 ± 0.1 mg/dl.

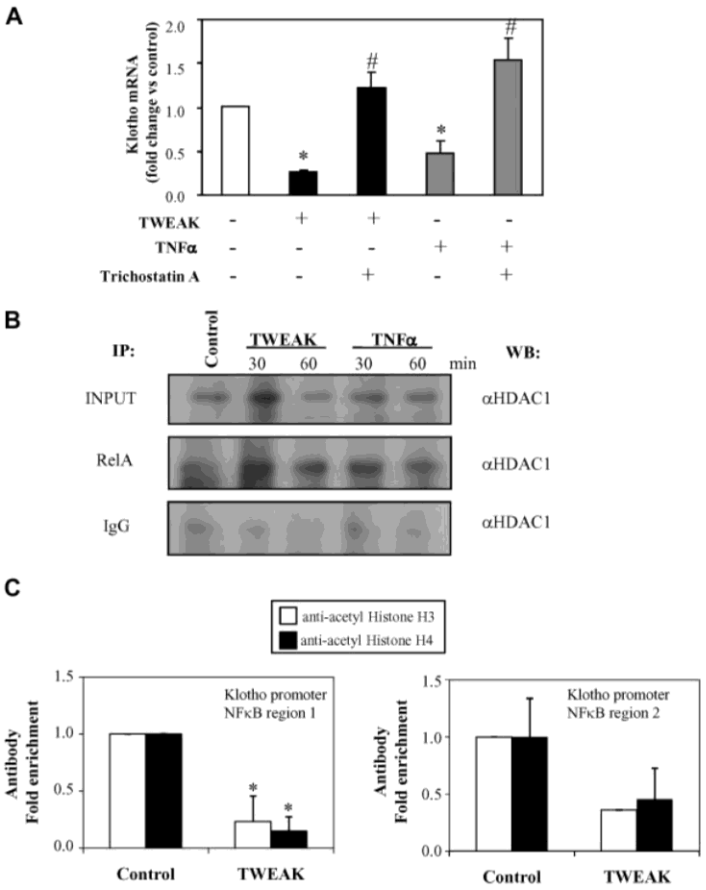

Figure 9. TWEAK- and TNFα-mediated downregulation of Klotho mRNA in cultured tubular cells requires HDAC activity. (A) The HDAC inhibitor TSA rescues TWEAK- or TNFα-induced repression of Klotho at 3 hours in MCT cells. Cells were prestimulated with TSA (100 ng/ml) 1 hour before addition of cytokines. Mean $\pm$ SD of three independent experiments. $^{*}P < 0.0001$ *versus* control; $^{\#}P < 0.001$ *versus* cytokine alone. (B) TWEAK and TNFα induce RelA association with HDAC1 in MCT cells. Cells were stimulated with TWEAK (100 ng/ml) or TNFα (30 ng/ml) for 30 and 60 minutes, to coincide with peak RelA nuclear translocation. Nuclear extracts were immunoprecipitated with 3 μg anti-RelA antibody or control IgG and then Western blotted with anti-HDAC1. Nuclear extract input was 25 μg. (C) TWEAK induces deacetylation at the Klotho promoter in MCT cells at 60 minutes as assessed by ChIP. Anti-acetylated histone H3 and H4 antibodies were used. The presence of acetylated histone H3 and H4 at the Klotho promoter was detected by RT-PCR with specific primers for two different NFκB binding sites of the Klotho promoter. Normal rabbit IgG was used as negative control for the specificity of the immunoprecipitation (intraperitoneally). As a positive control, aliquots of chromatin fragments obtained before intraperitoneally were also subjected to RT-PCR analysis (Input). Immunoprecipitated DNA with histone modification was normalized to a 100-fold dilution of input chromatin. Data are expressed as fold enrichment of RelA binding compared with negative control antibody (normal rabbit IgG). $n = 4$; $^{*}P < 0.04$ *versus* control.

ChIP assay

ChIP assays using 0.5 to 1 $\times$ 10^6 cells per sample were performed as described previously[61] using 10 μg/ml anti-AcH3, anti-AcH4, and anti-RelA (Upstate Biotechnology) antibodies. Normal IgG was used as negative control. In brief, cells fixed with 1% formaldehyde were lysed in SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and sonicated. Chromatin was diluted into ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and incubated with the antibodies overnight at 4°C. Antibody-chromatin complexes were precipitated with salmon sperm DNA/Protein A–Agarose beads (Upstate Biotechnology), washed, and eluted from the beads using elution buffer (1% SDS, 0.1 M $NaHCO_3$). After crosslink reversal and proteinase K treatment, DNA was extracted with phenol-chloroform, and ethanol was precipitated. DNA immunoprecipitated from 1 μl eluted DNA was analyzed in duplicate by real-time PCR. Primers were designed to amplify specific NFκB regions (regions 1 and 2) at promoter of the Klotho gene, identified by TFSEARCH: Searching Transcription Factor Binding Sites (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html). Primers for each promoter are listed in Supplemental Table S1. Aliquots of chromatin obtained before immunoprecipitation were analyzed as input control. Results are presented as fold enrichment of precipitated DNA associated with a given histone modification or RelA binding, relative to a 1/100 dilution of input chromatin.

siRNA Transfection

Cells were seeded in six-well plates and transfected on the following day with 20 nM siRNA using Lipofectamine 2000 reagent (Invitrogen).[62] After 48 h, the transfected cells were harvested for Western blot or PCR analysis. siRNA were synthesized by Santa Cruz Biotechnology.

Statistical Analysis

Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as mean $\pm$ SD. Significance at the $P < 0.05$ level was assessed by *t* test for two groups of data and ANOVA for three or more groups.

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DISCLOSURES

None.

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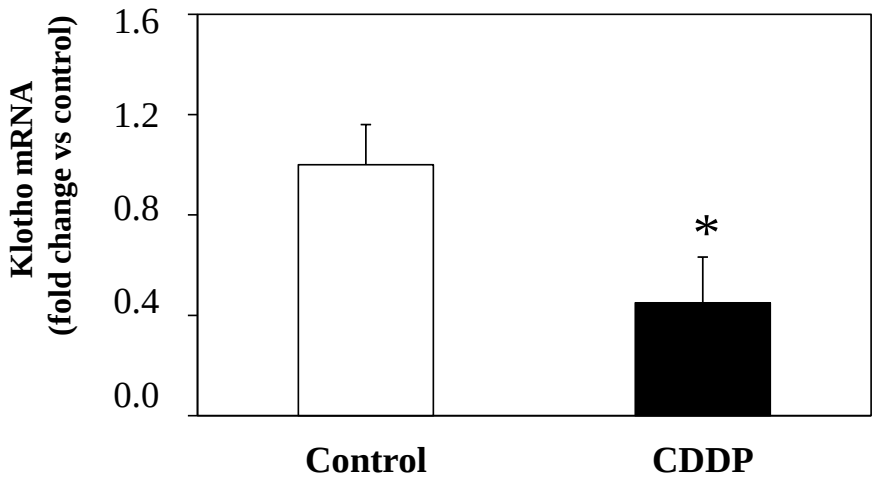
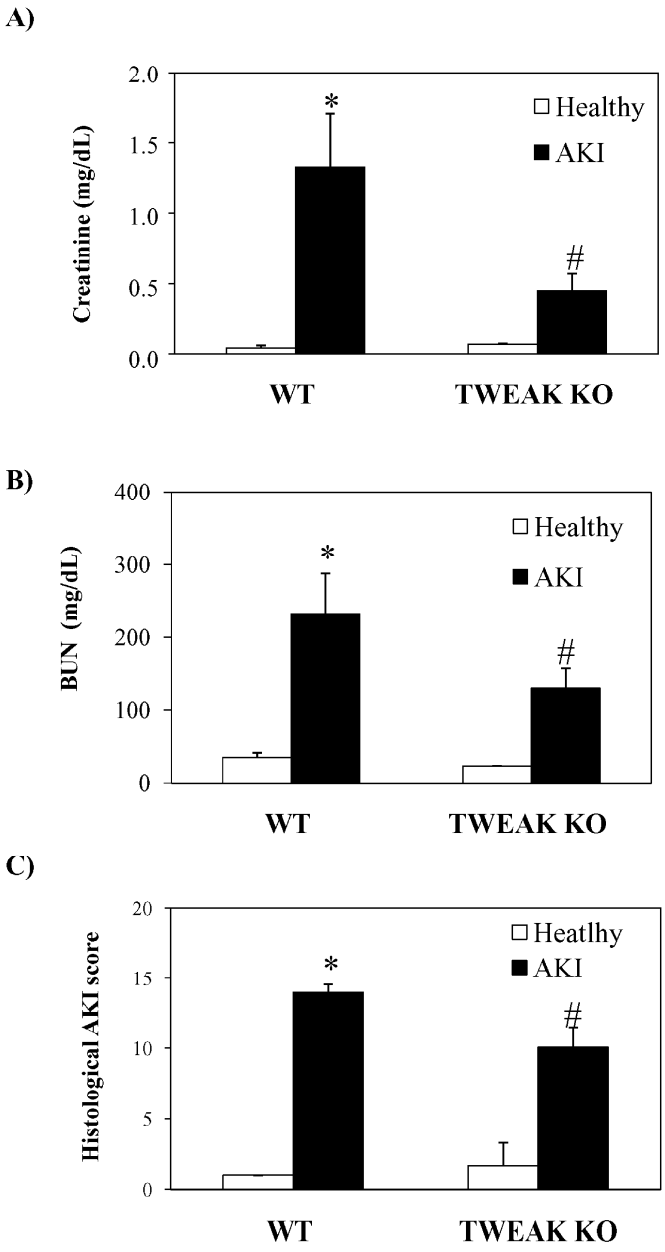
Supplemental information for this article is available online at http://www.jasn.org/.

Supplementary Figure 1



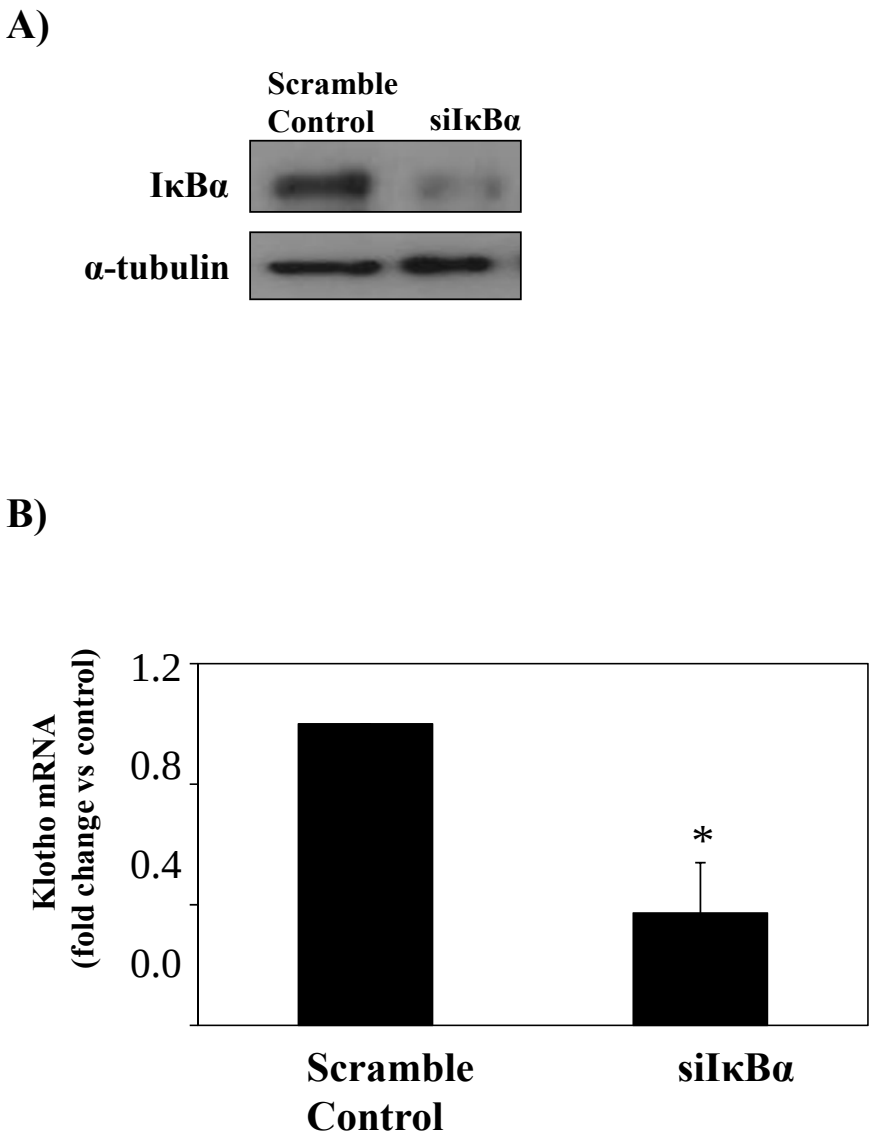
Supplementary figure 1. Kidney Klotho expression is decreased in AKI induced by cisplatin (CDDP). Mice were injected with a single dose of CDDP or vehicle (control) and killed at 72 h. Total kidney Klotho mRNA levels were measured by qRT-PCR. Mean±SEM of 5 animals per group. *p<0.05 vs control.

Supplementary Figure 2



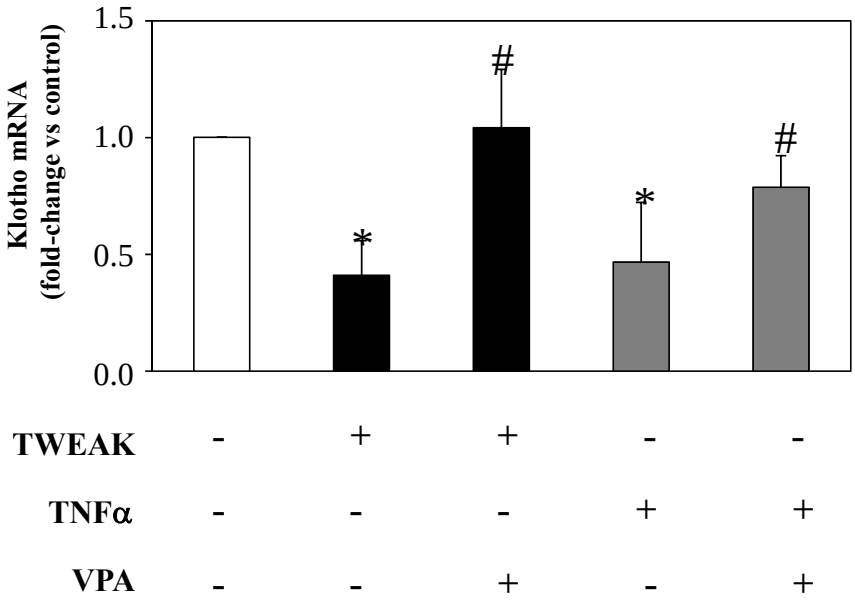
Supplementary figure 2. TWEAK absence improves renal function and reduces tubular injury during AKI. AKI was induced by a folic acid overdose in healthy WT or TWEAK KO mice. **A, B)** Renal function was assessed at 72 h measuring plasma creatinine (**A**) and BUN (**B**). Mean (±SEM) of 5 animals per group. *p<0.005 vs healthy WT mice #p<0.04 vs AKI WT mice. **C)** Histological tubular injury was assessed by an AKI score which had a maximum value of 21. Mean ±SEM of 5 animals per group. *p<0.005 vs healthy WT mice #p<0.03 vs AKI WT mice.

Supplementary Figure 3



Supplementary figure 3. IκBα down-regulation by siRNA reduces Klotho expression in tubular cells. A) IκBα protein level is efficiently down-regulated by a specific siRNA. Tubular cells were transfected with a siRNA oligonucleotide targeting IκBα or scrambled control and IκBα expression was assessed 48 hours after transfection by Western blot. **B)** Klotho mRNA expression in tubular cells transfected 48 h earlier were quantified by qRT-PCR. IκBα binds RelA and keeps it inactive in the cytoplasm. IκBα siRNA targeting, a maneuver facilitating RelA nuclear translocation and NFkB activation, reduces Klotho mRNA in unstimulated cells. Mean ±SD of three independent experiments. *p<0.02 vs scrambled control.

Supplementary Figure 4



Supplementary figure 4. The HDAC inhibitor valproic acid prevents TWEAK- and TNFα-induced downregulation of Klotho mRNA in cultured tubular cells. A) The histone deacetylase inhibitor valproic acid (VPA) rescues TWEAK or TNFα-induced repression of Klotho at 3h in MCT cells. Cells were pre-stimulated with VPA (3 mM) 1 hour prior to cytokines. Mean±SD of three independent experiments. *p<0.005 vs control. #p<0.05 vs cytokine alone.

IV. DISCUSIÓN

El FRA es un síndrome caracterizado por un daño tubular y un descenso drástico y brusco de la filtración glomerular. La actual comprensión de la fisiopatología del FRA parenquimatoso es incompleta y por esto no hay un tratamiento específico. Aunque la supervivencia ante un FRA aislado ha mejorado con los recientes avances en la terapia del reemplazo renal, la mortalidad del FRA complicado por una disfunción multiorgánica se ha mantenido sin cambios y se estima en aproximadamente el 50% [307].

El FRA no es un evento aislado, sino que a través de un mecanismo inflamatorio que implica la migración de neutrófilos, la expresión de citoquinas y el aumento del estrés oxidativo da lugar a la disfunción de múltiples órganos tales como el corazón, los pulmones, el hígado, los intestinos y el cerebro [307]. Además el FRA contribuye a la progresión de la ERC y la ERC predispone al FRA.

Por ello, es necesario desarrollar terapias que puedan modular o frenar el desarrollo de esta enfermedad. En esta tesis hemos demostrado que las citoquinas inflamatorias pueden regular la inflamación túbulointersticial, así como la inflamación asociada al envejecimiento, tanto promoviendo la expresión de factores proinflamatorios como limitando la expresión de mediadores antiiflamatorios, y que estos dos efectos tienen lugar a través de la activación del factor de transcripción NF-κB. Estas observaciones podrían ser de gran utilidad clínica para el diseño de nuevos abordajes terapéuticos del FRA y de la ERC.

1. TWEAK PROMUEVE LA EXPRESIÓN TUBULAR DE CXCL16: POSIBLE CONTRIBUCIÓN A LA INFLAMACIÓN TÚBULOINTERSTICIAL RENAL

Uno de los principales hallazgos de este estudio es que TWEAK aumenta la expresión de CXCL16 en las células tubulares renales, tanto en cultivo como *in vivo,* a través de la activación del factor de transcripción NF-κB y que CXCL16 coopera con TWEAK en promover la respuesta inflamatoria de las células tubulares. La neutralización de TWEAK disminuye la expresión de CXCL16 y la infiltración de linfocitos CD3 en el modelo experimental de inflamación túbulointersticial, lo que sugiere la relevancia de esta observación en la lesión renal. En este sentido, también se observa que CXCL16 y Fn14 están presentes en las células tubulares de muestras renales humanas con inflamación túbulointersticial.

Existen evidencias de que CXCL16 participa en la patología renal. El bloqueo de CXCL16 en la fase inflamatoria aguda de la glomerulonefritis experimental atenúa significativamente la infiltración de monocitos/macrófagos y el daño glomerular y tubular [75;302;303]. Sin embargo, sigue habiendo lagunas con respecto a la regulación de la expresión de CXCL16 y sus acciones sobre las diferentes células renales intrínsecas. En el riñón, CXCL16 se expresa constitutivamente en las células mesangiales, podocitos y células tubulares humanas [90;251;252]. La expresión de CXCL16 glomerular está aumentada en la nefropatía membranosa humana y en la lesión glomerular experimental [90]. En las células

glomerulares cultivadas, la expresión de CXCL16 aumenta por TNFα e IFNγ, pero poco se sabe sobre los factores que regulan la expresión de CXCL16 en las células tubulares renales [90;251;252]. IFNγ aumenta la expresión de CXCL16 en las células primarias de la rama gruesa ascendente y en las células tubulares distales [251]. Sin embargo, hay evidencia de una regulación diferencial de la expresión de CXCL16 dependiendo del segmento tubular y de la etiología de la lesión tubular. El aumento de la expresión de CXCL16 en aloinjertos de FRA humanos se observa de manera específica en la región apical del túbulo [251]. Sin embargo, la expresión de CXCL16 tubular en los pacientes con rechazo intersticial y prominente infiltración de células T fue mayoritariamente negativa [251]. Esta baja expresión en la membrana basolateral de CXCL16 en el rechazo intersticial se atribuyó a un aumento en la liberación de CXCL16, por lo que funcionaría como quimioquina y atraería también linfocitos T. En nuestro modelo experimental de inflamación túbulointersticial nefrotóxico apreciamos una expresión tanto apical como basolateral de CXCL16. Estos patrones no se solapan en la mayoría de los túbulos. Así CXCL16 derivado de las células tubulares podría actuar sobre otras células tubulares adyacentes o a través de la luz tubular y sobre el intersticio.

Las quimioquinas reclutan leucocitos, los cuales tienen un papel clave en el daño tisular túbulointersticial renal durante el FRA y ERC [202;262]. CXCL16 desempeña un papel esencial en el reclutamiento de células T [90;251;252] y, recientemente, se ha demostrado que TWEAK induce infiltración renal de células T [74]. En este trabajo, se demuestra que TWEAK regula la expresión de CXCL16 en las células epiteliales tubulares renales en cultivo e in vivo. Además de aumentar la expresión renal de CXCL16, TWEAK aumenta el número de linfocitos CD3 intersticiales. De esta manera, la neutralización de TWEAK disminuyó la infiltración de linfocitos CD3. Aunque TWEAK regula la expresión de quimioquinas, nosotros hipotetizamos que la expresión de CXCL16 en células tubulares puede contribuir al reclutamiento de células T, como los estudios in vitro sugieren, y colabora con TWEAK en promover la inflamación. En este sentido, en la inflamación intersticial humana, los túbulos que expresaron Fn14 también expresaron CXCL16 y estaban rodeados de infiltrado inflamatorio.

Curiosamente, se ha observado que la excreción urinaria de CXCL16 aumenta en pacientes y ratones con nefritis lúpica, al igual que sucede con los niveles urinarios de TWEAK [254;303]. A este respecto, la expresión de CXCL16 aumenta en los glomérulos y en los túbulos de ratones con lupus y en la nefritis anti-MBG experimental [75;302;303]. Por otra parte, se observaron niveles elevados de CXCL16 urinario en pacientes trasplantados con disfunción renal aguda, en los cuales sólo el CXCL16 tubular estaba aumentado, lo que sugiere un origen tubular del CXCL16 urinario [251;303]. Así, la inducción de la expresión de CXCL16 por TWEAK en las células tubulares podría contribuir a estas observaciones.

CXCL16 tiene actividades biológicas más allá de la quimiotaxis de leucocitos y estas actividades pueden variar de manera célula específica. CXCL16 induce la proliferación de células precursoras gliales, fibroblastos miocárdicos, células trofoblásticas, células endoteliales, células musculares lisas de aorta y

células mesangiales [33;48;96;113;252;315]. Sin embargo, en células cancerosas, CXCL16 soluble induce la proliferación del tumor mientras que CXCL16 transmembrana la suprime [55]. En células renales epiteliales (células tubulares y podocitos) CXCL16 promueve la quimiotaxis de células T y puede regular la captación de LDLox [90], mientras que en células mesangiales CXCL16 promueve la proliferación celular [252]. A pesar de este papel regulador de la proliferación en células mesenquimales, endoteliales y tumorales, CXCL16 no induce proliferación ni apoptosis de células epiteliales tubulares renales murinas, ni solo ni en combinación con TWEAK. TWEAK, por su parte, induce la proliferación del epitelio tubular renal no estresado [244] y la apoptosis en células estresadas por un ambiente inflamatorio compuesto por TNFα/IFNγ [126]. A pesar de la falta de un efecto de CXCL16 sobre la proliferación celular tubular, las células tubulares expresan el receptor CXCR6 y CXCL16 tiene un efecto proinflamatorio en las células tubulares aumentando la expresión génica de ICAM-1, MCP-1 y RANTES inducida por TWEAK. TWEAK promueve la expresión de quimioquinas a través de dos vías distintas de NF-κB, la vía canónica y la no canónica [246;249]. La evidencia presentada en este trabajo indica que TWEAK promueve la expresión de CXCL16 a través de la vía canónica de NF-κB (sensible a partenolide) lo que amplía el rango de quimioquinas reguladas por TWEAK a través de esta vía. En este sentido, las quimioquinas inducidas por TWEAK pueden comportarse de manera coordinada para atraer a las células T CD3 y otros leucocitos al espacio tubulointersticial. No podemos excluir que CXCL16 tenga acciones adicionales en las células tubulares, en relación con su papel de receptor basurero [258].

Como resumen de esta primera parte, nuestros resultados identifican a TWEAK como un nuevo regulador de la expresión de CXCL16 en las células tubulares en cultivo e *in vivo*. Así se amplía el rango de mediadores inflamatorios que están regulados directamente por TWEAK. Asimismo, hemos identificado acciones proinflamatorias directas de CXCL16 en las células tubulares renales (**Figura 7**).

Estos datos serán útiles para definir nuevas estrategias terapéuticas dirigidas a TWEAK, NF-κB y/o CXCL16 en la enfermedad renal.

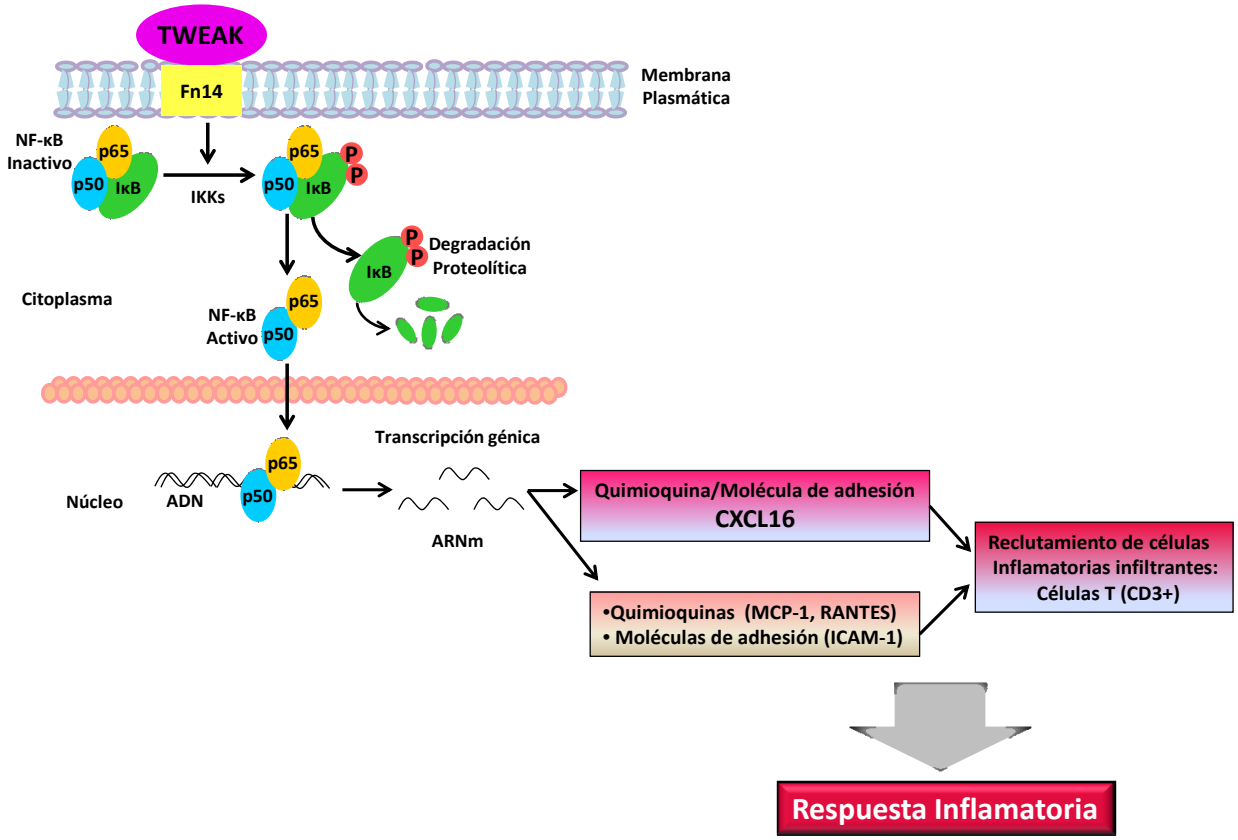


Figura 7. ***Esquema representativo de la regulación de la expresión de CXCL16 y de la respuesta inflamatoria inducida por TWEAK en la célula tubular renal.***

2. LA INFLAMACIÓN REGULA LA EXPRESIÓN TUBULAR DE KLOTHO

El otro descubrimiento principal de este estudio es que la inflamación disminuye la expresión de Klotho en células tubulares renales a través de un mecanismo dependiente de NF-κB RelA. En concreto citoquinas inflamatorias individuales tales como TWEAK o TNFα disminuyen Klotho tanto en cultivo celular como *in vivo*. Ambas citoquinas proinflamatorias aumentan la expresión de una variedad de mediadores inflamatorios a través de NF-κB RelA[27;242]. Sin embargo, en el caso de Klotho, NF-κB RelA parece reducir su expresión génica en un ambiente inflamatorio. Estos hallazgos pueden tener implicaciones terapéuticas en la lesión renal y en la inflamación asociada al envejecimiento prematuro.

El riñón es el principal sitio de expresión de Klotho. En la ERC en general y durante el daño renal por hipertensión, diabetes [4] o FRA experimental inducido por isquemia-reperfusión [271], la expresión renal de Klotho disminuye, lo que también se corroboró con nuestros resultados. De acuerdo con estos datos, podemos especular que una disminución en la expresión renal de Klotho puede tener consecuencias sistémicas y locales de tipo adverso. Con respecto a los efectos sistémicos potenciales, la disminución de la expresión renal de Klotho durante el FRA nefrotóxico se mantiene más allá de la recuperación de la función renal y se asocia con una disminución del Klotho circulante. La disminución de Klotho renal, circulante y excretado en la orina fue recientemente mostrada en el FRA inducido por isquemia-reperfusión [112]. Los pacientes con ERC tienen un daño progresivo de múltiples órganos, entre los cuales se encuentra el sistema cardiovascular, dando lugar a una tasa de mortalidad diez veces mayor que la de los controles de la misma edad, lo que significa, un envejecimiento acelerado [266;267]. Curiosamente, la inflamación sistémica también promueve la morbilidad y mortalidad cardiovascular, y se ha asociado también con otras enfermedades dependientes de la edad, tales como la osteoporosis y la demencia [218;229]. A nivel local, una reducción de la expresión de Klotho contribuye a la progresión de la insuficiencia renal, mientras que su sobreexpresión mejora la lesión renal en un modelo murino [94]. La transferencia génica de Klotho mediada por adenovirus mejora la creatinina sérica y reduce la apoptosis renal inducida por isquemia-reperfusión en ratas [268;271]. La transfección de Klotho reduce la apoptosis de células tubulares inducida por H_2O_2 en cultivo [175]. Klotho también se puede expresar en células extrarrenales. Así, la expresión de Klotho se reduce en los linfocitos CD4 de pacientes de edad avanzada [301].

Nuestros resultados muestran que TWEAK y TNFα inducen la disminución de Klotho de manera dosis dependiente en células cultivadas, que TWEAK disminuye la expresión de Klotho en los riñones *in vivo* y que el bloqueo de TWEAK impide la disminución de Klotho durante el daño renal. Estas observaciones están en línea con observaciones publicadas de que Klotho renal se redujo en el curso de la inflamación sistémica causada por una enfermedad inflamatoria intestinal y un anticuerpo

neutralizante anti-TNF atenuó la inflamación intestinal y revocó la represión de la expresión renal de Klotho [275]. En este estudio hemos identificado a NF-κB, y en concreto a homo o heterodimeros de NF-κB que contienen la subunidad RelA en la represión de la expresión de Klotho. En concreto, las evidencias siguientes sugieren que la activación de RelA es necesaria para la represión de Klotho inducida por TWEAK y TNFα:

1) La inhibición de IκBα por el siRNA, es una maniobra que permite la translocación de RelA al núcleo, lo que promueve la disminución de Klotho.

2) Un inhibidor de la activación de RelA, partenolide, previene la disminución de Klotho inducida por TWEAK y TNFα.

3) El patrón temporal de la represión de Klotho observado es similar al de la activación de RelA inducida por TWEAK y TNFα.

4) Y RelA se une al promotor de Klotho en células tubulares murinas estimuladas con TWEAK [99;242].

La disminución de la expresión génica mediada por NF-κB se había observado previamente en células estimuladas con TNFα. Por ejemplo, TNFα disminuye la expresión génica de la argininosuccinato sintasa, la óxido nítrico sintasa endotelial y la proteína morfogenética ósea-4 [7;81;314]. Sin embargo, no se había observado que TWEAK disminuyera la expresión génica mediada por NF-κB. A diferencia de TNFα, TWEAK activa a las 24 horas un subconjunto del complejo proteico de NF-κB2 (vía no canónica de activación de NF-κB) [232;246]. Sin embargo, TWEAK y TNFα comparten la activación temprana, por la via canónica, de los complejos de NF-κB que contienen Rel A [232;242;246]. El hecho de que tanto TWEAK como TNFα disminuyan Klotho de forma temprana argumenta en contra de una participación de NF-κB2, que sólo se activa por TWEAK y que tendría lugar después de que los cambios en el ARNm de Klotho ya han ocurrido.

La liberación de NF-κB de las proteínas inhibidoras IκBs y su posterior translocación nuclear son sólo los acontecimientos iniciales que conducen a la inducción o represión de los genes regulados por NF-κB. Una vez en el núcleo, NF-κB debe tener acceso a sus sitios afines en los genes diana. Mientras que algunos sitios se encuentran de manera accesible constitutivamente muchos otros están asociados con las histonas nucleosomales de manera que impiden la unión de NF-κB. La unión a estos sitios requiere una remodelación específica de la cromatina impulsados por la cooperación funcional de los factores de transcripción [190]. Además de los factores específicos genéticos hay factores específicos de estímulo que determinan la actividad inductora o represora de NF-κB sobre la transcripción génica [28]. Así, NF-κB inducido por estímulos citotóxicos, no citoquinas, es funcionalmente distinto al inducido por estímulos inflamatorios, tales como TNFα: los primeros reprimen la expresión génica antiapoptótica,

mientras que los segundos la promueven [28]. Hay diferentes mecanismos de la represión génica dependiente de p65/RelA. Recientemente, se ha puesto de manifiesto la importancia de la actividad de HDAC. La actividad de HDAC es necesaria para la represión de PPARδ dependiente de p65/RelA de los queratinocitos humanos, de los genes antiapoptóticos de los fibroblastos y del factor de crecimiento derivado de las plaquetas de las células del músculo liso [1;28;145]. Hemos observado que TWEAK y TNFα aumentan la asociación de RelA con HDAC1 en el núcleo al mismo tiempo que disminuyen la expresión de Klotho en las células tubulares. Este resultado sugiere que la disminución de Klotho inducida por TWEAK y TNFα podría estar mediada por la actividad de HDAC. En este sentido, observamos que dos inhibidores de HDAC, tricostatina A y el acido valproico, evitaron la represión de Klotho inducida por TWEAK o TNFα. Además, TWEAK induce la deacetilación de las histonas H3 y H4 en el promotor de Klotho en las células tubulares renales murinas.

En resumen, las citoquinas inflamatorias TWEAK y TNFα disminuyen Klotho en las células tubulares renales a través de un mecanismo dependiente de NF-κB (**Figura 8**). Estos datos son de interés para la lesión renal y el envejecimiento acelerado. Nuestros resultados pueden ser relevantes para diseñar estrategias terapéuticas que regulen la expresión Klotho enfocado a la manipulación terapéutica de la actividad de citoquinas inflamatorias o de NF-κB.

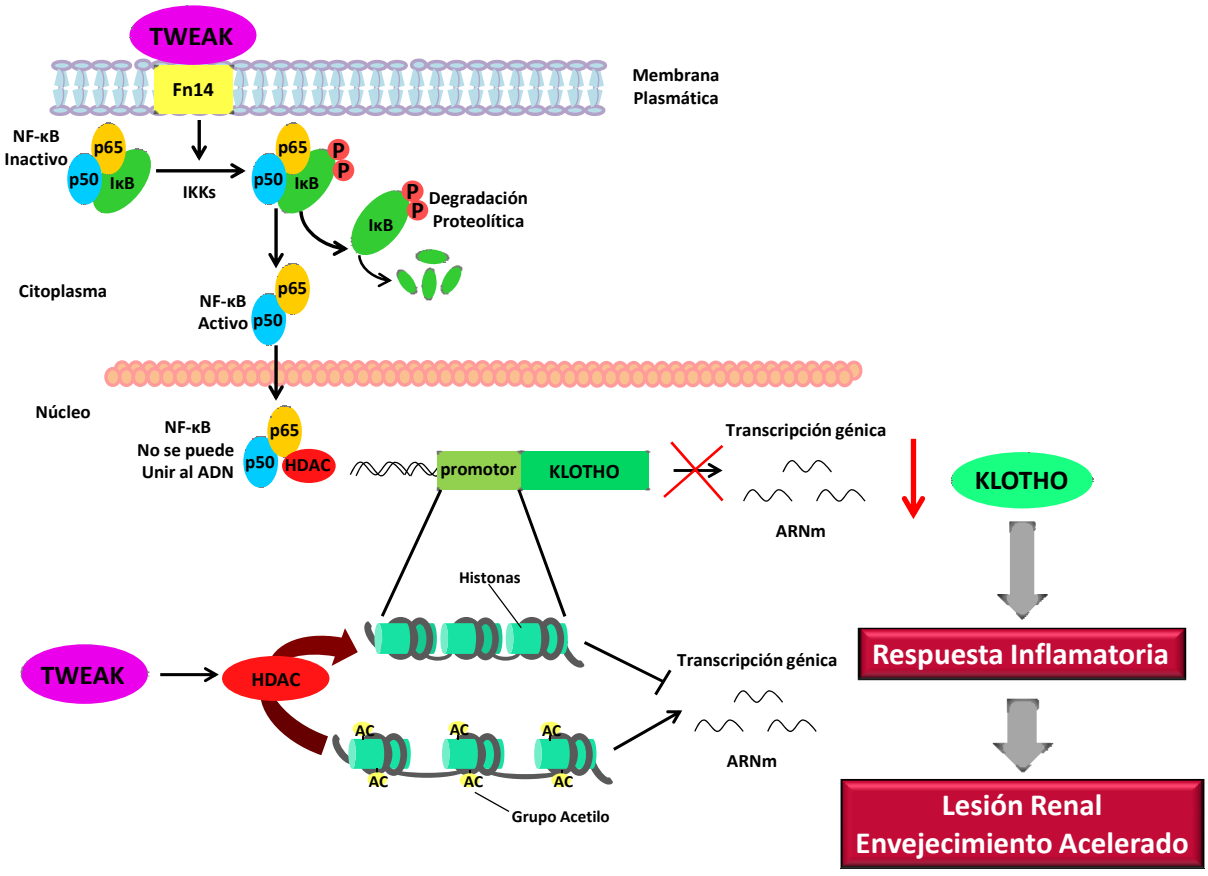


Figura 8. ***Esquema representativo de la inhibición de la expresión de Klotho en la célula tubular renal.***

V. CONCLUSIONES

1. La expresión de CXCL16 aumenta y la de Klotho disminuye en el daño renal agudo y ambas se correlacionan con la expresión de Fn14.

2. TWEAK aumenta la expresión de CXCL16 y disminuye la de Klotho en las células tubulares renales cultivadas e *in vivo* a través de NF-κB, tanto en riñones sanos como durante el fracaso renal agudo.

3. CXCL16 tiene acciones proinflamatorias en las células tubulares renales.

4. Estos estudios identifican a TWEAK/Fn14 y NF-κB como factores claves en el fracaso renal agudo, con capacidad tanto para aumentar la expresión de mediadores proinflamatorios, como CXCL16, como para disminuir la de factores antiinflamatorios y citoprotectores como Klotho.

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VII. ANEXO A

Los resultados presentados en esta tesis han sido publicados parcialmente en:

Artículos originales:

- **Izquierdo MC**, Sanz AB, Mezzano S, Blanco J, Carrasco S, Sanchez-Niño MD, Benito-Martín A, Ruiz-Ortega M, Egido J, Ortiz A. TWEAK (tumor necrosis factor-like weak inducer of apoptosis) activates CXCL16 expression during renal tubulointerstitial inflammation. *Kidney Int*. 2012 Jun; 81(11):1098-107. doi: 10.1038/ki.2011.475. Epub 2012 Jan 25.

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Revisiones:

- **Izquierdo MC**, Perez-Gomez MV, Sanchez-Niño MD, Sanz AB, Ruiz-Andres O, Poveda J, Egido J, Ortiz A. Klotho, phosphate and inflammation/aging in chronic kidney disease. *Nephrol Dial Transplant.* (Aceptado).

- **María Concepción Izquierdo**, Ana B. Sanz, María Dolores Sanchez-Niño, María Vanessa Perez-Gomez, Marta Ruiz-Ortega, Jonay Poveda, Olga Ruiz-Andres, Adrian M. Ramos, Juan A. Moreno, Jesús Egido1, Alberto Ortiz1. Acute kidney injury transcriptomics unveils a relationship between inflammation and ageing –inflamm-aging. *Nefrologia*. (Aceptado)

Otros trabajos publicados durante el desarrollo de esta tesis:

- Caroline Y. Hu, Davoud Mohtat, Yiting Yu, Sanchari Bhattacharya, Yi-An Ko, **Maria C. Izquierdo**, Krishna Gundabolu, Kristin Ware, Tushar Bhagat, Christoph Heuck, Meher Walia,Junaid Saleh, Masako Suzuki, James Pullman, Shirley Liu, John Greally, Katalin Susztak, Amit Verma. Renal carcinoma is characterized by widespread aberrant methylation that targets regulatory regions of the kidney genome. *Genome Biology.* (En revision).

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- **Izquierdo, M.C.**; Sanz, A.B.; Carrasco, S.; Ruiz-Ortega,M.; Egido, J.; Ortiz, A. TWEAK regulates CXCL16 expression in renal tubular cells. Poster. 43rd Annual Meeting and Scientific Exposition. American Society of Nephrology. Denver 2010.

VIII. ANEXO B

Klotho, phosphate and inflammation/aging in chronic kidney disease

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Abstract

Evidence is emerging for the inflammatory nature of many aging-associated diseases, including atherosclerosis, vascular calcification, diabetes and chronic kidney disease (CKD), among others. Aging itself results in chronic low grade inflammation that promotes end-organ damage. Inflammatory organ damage, in turn, may contribute to inflammation. Recent research has identified the kidney-secreted hormone Klotho as a central player at the ageing-inflammation interface. Thus, systemic or local renal inflammation decreases kidney Klotho expression. Klotho downregulation may be induced by specific cytokines such as TNFα or TWEAK through the canonical activation of the inflammatory transcription factor NFκB and, specifically RelA. In addition inflammatory cytokines lead to the epigenetic inactivation of Klotho transcription. Klotho itself has antioxidant and anti-inflammatory properties and the canonical NFκB component RelA is one of its targets. Klotho is a key regulator of phosphate balance and a role of phosphate in aging has been shown. However, the potential relationship between phosphate and inflammation requires further clarification. A correct understanding of these interactions may lead to the design of novel therapeutic approaches to CKD and CKD-related inflammatory and aging features as well as to inflammation/aging in general.

Key words: Ageing, Klotho, Inflammation, NF-kappaB, Phosphate

Many aging-associated diseases, including atherosclerosis, vascular calcification, osteoporosis, osteoarthritis, diabetes and chronic kidney disease (CKD) have an inflammatory nature. Aging itself results in chronic low grade inflammation that promotes end-organ damage. Inflammatory organ damage, in turn, may contribute to aging. CKD has many features of an accelerated aging syndrome, including accelerated vascular disease, bone disease and an increase in age-adjusted total and cardiovascular death risk [316]. Chronic inflammation is a recognized feature of CKD that impinges on prognosis [316]. Hyperphosphatemia or a positive phosphate balance are also

recognized features of CKD. Derangements of phosphate metabolism are associated with aging features such as vascular calcification and death in CKD. Furthermore, this relationship may extend beyond CKD. In a community-based cohort of 3015 healthy young adults, phosphorus levels at a mean age of 25 years were significantly associated with coronary artery calcium 15 years later [70]. In addition, in CKD patients not on dialysis serum phosphate was independently associated with inflammatory markers such as IL-6 and it was hypothesized that elevated serum phosphate might play a role in the development of inflammation in CKD [194]. Recent advances have provided new insights into the relationship between CKD, phosphate, inflammation and aging. A kidney protein, Klotho (α-Klotho), may link aging, phosphate metabolism and inflammation. Klotho deficiency is associated with premature aging in mice [135]. The best characterized function of Klotho is regulation of phosphate metabolism [108]. Interestingly, hyperphosphatemia and low urinary phosphate excretion are features of the human Hutchinson-Gilford progeria syndrome of premature aging [204]. Additionally, in mice the induced expression of the most common progeria mutation (LMNA p.G608G) resulted in upregulation of multiple genes in major inflammatory pathways [227]. This points to a relationship between aging, phosphate and inflammation that may explain some of the features of CKD. We now discuss recent evidence linking bidirectionally Klotho with inflammation and its relevance for CKD.

Klotho structure and function

The Klotho (kl) gene, named after the Greek goddess who spins the thread of life, was identified in 1997 as the gene disrupted in mice with a syndrome resembling human premature ageing syndromes [135]. Klotho is mainly expressed in the kidney, the brain (in the choroid plexus), the parathyroid gland, and the skeletal muscle. In the kidney, distal convoluted tubule are the main sites of Klotho expression [135].

Two Klotho gene transcripts are generated through alternative transcriptional termination, encoding a membrane or a secreted soluble protein [108]. In humans the expression level of the secreted isoform predominates over the membrane isoform, whereas it is viceversa in mice. Membrane Klotho is a single-pass transmembrane protein of 135 kDa. The membrane form of Klotho consists of an extracellular domain composed of two internal repeats (KL1 and KL2) that share a homologous amino acid sequence to β-glucosidase, a transmembrane domain and a short intracellular domain. ADAM10 and ADAM17 may release soluble Klotho composed of two internal repeats (KL1 and KL2), while soluble Klotho encoded by the

alternative transcript only contains KL1. Thus, there are two different origins for soluble Klotho: alternative transcripts and proteolytic processing of the membrane form. Transmembrane Klotho is a co-receptor for FGF23, conferring FGF23-specificty and high affinity to FGFR1c, 3c and 4. In addition, the secreted Klotho protein can regulate multiple growth factor signaling pathways, including insulin/IGF-1, Wnt and TGFβ1, and the activity of multiple ion channels and transporters. These effects are thought to result from the enzymatic glycosidase activity that modifies the sugar composition of proteins regulating their activity and/or cell surface retention time. Klotho also protects cells and tissues from oxidative stress.

One key action of FGF23/Klotho in protection from phosphate loads. Thus, in proximal tubular cells FGF23 down-regulates the production of 1,25 $(OH)_2$ vitamin D by inhibiting the expression of 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) and stimulating 24-hydroxylase (CYP24A1) and decreases the phosphate reabsorption transporter NaPi-IIa at the apical plasma membrane [220]. In addition, the glycosidase activity of soluble Klotho decreases the activity of NaPi-IIa [110]. Thus, FGF-23/Klotho decreases tubular phosphate reabsorption, causing phosphaturia, and decreases vitamin D-stimulated intestinal phosphate absorption. Both actions tend to decrease phosphate load. In addition Klotho regulates calcium metabolism.

Premature aging in Klotho deficient mice

FGF23 and Klotho deficiency have similar phenotypes characterized by hyperphosphatemia, hypercalcemia and elevated calcitriol levels [220]. Interestingly, despite the recognized anti-inflammatory actions of VDR activation [238], animal models characterized by Klotho or FGF-23 deficiency or FGF-23 immunoneutralization illustrate the dangers of continuous VDR activation in face of hyperphosphatemia, leading to increased mortality.

Klotho-/- mice have a short lifespan (average 60 days) as compared wild-type mouse living for approximately 3 years. In addition they show numerous physical, biochemical, and morphological features consistent with premature aging, including kyphosis, uncoordinated movement, hypogonadism, infertility, severe skeletal muscle wasting, emphysema, and osteopenia, as well as generalized atrophy of the skin, intestine, thymus, and spleen [198]. Around 4 weeks ectopic calcification (organs and arterial walls) develop and progress gradually with age. Hypercalcemia and hyperphosphatemia are noted as well as renal failure characterized by kidney calcification and an increased apoptotic rate.

Phosphate appears to be key in defining the Klotho-/- phenotype. Increased renal activity of sodium-phosphate cotransporters (NaPi2a) leads to severe hyperphosphatemia. Genetically reducing serum phosphate levels in Klotho(-/-) mice by generating a NaPi2a and Klotho double-knockout (NaPi2a(-/-)/Klotho(-/-)) strain ameliorated premature aging-like features and prolonged survival. However, when hyperphosphatemia was induced in NaPi2a(-/-)/Klotho(-/-) mice by feeding with a high-phosphate diet, premature aging-like features reappeared and lifespan was shortened [198].

Klotho and inflammation

There is a bidirectional relationship between Klotho and inflammation. On one hand, inflammation downregulates Klotho expression. On the other, Klotho downregulates inflammation**.**

Both systemic and local inflammation may decrease kidney Klotho expression. Thus, kidney Klotho expression is down-regulated in rats with lipopolysaccharide-mediated acute inflammatory stress but not by acute hypovolemic stress [199]. Renal Klotho was reduced in mice with different forms of inflammatory bowel disease and a neutralizing anti-TNFα antibody attenuated inflammation and reversed the repression of Klotho expression [276].

In addition, kidney Klotho is reduced in chronic or acute experimental models of renal injury characterized by renal inflammation. In diabetic nephropathy decreased kidney Klotho expression can be reversed by an antioxidant without changing blood glucose levels [38;312]. Mice with CKD induced by uninephrectomy plus ischemia-reperfusion injury in contralateral kidney had very low renal, plasma, and urinary levels of Klotho [111]. Urinary Klotho decreased in human stage I CKD, a stage characterized by preserved renal function but some evidence, usually albuminuria, of kidney injury [111]. This concept is interesting because in this stage there is barely any loss of tubular cells that may justify a decrease in Klotho. Rather, the main histological picture is mild interstitial inflammation as a result of albuminuria or the cause of kidney injury. We hypothesize that inflammation may be the driver for decreased Klotho in early CKD. By contrast to urine, there are conflicting reports on serum Klotho in human CKD.

Ischemia reperfusion-induced acute kidney injury (AKI) in rodents reduced Klotho in the blood, urine, and kidneys and reduced urinary Klotho was observed in human AKI [112]. Reduced Klotho was also observed in nephrotoxic AKI in mice [178]. In this model TWEAK was a key contributor to reduced kidney Klotho both in

healthy mice and in AKI. Thus, TWEAK targeting by neutralizing antibodies or in TWEAK KO mice preserved kidney Klotho expression and circulating Klotho levels and also preserved renal function [178]. In renal tubular cells Klotho is downregulated by inflammatory cytokines such as TWEAK or TNFα that decreased Klotho gene transcription [178;276]. Increased expression of inducible nitric oxide synthase (iNOS) and NO had a key role in decreased Klotho gene transcription. The transcription factor NFκB RelA was required for suppression of Klotho gene transcription [178]. In addition, evidence was uncovered for epigenetic modulation of Klotho gene expression by inflammatory cytokines. TWEAK induced histone H3 and H4 deacetylation at the murine Klotho promoter in renal tubular cells and HDAC inhibitors preserved Klotho expression [178].

Klotho is an anti-inflammatory modulator in the kidney. In a mouse model of diabetes, Klotho depletion contributes to increase inflammation. Both soluble or membrane Klotho negatively regulates NFκB activation via a mechanism that involves RelA (Ser)[536] phosphorylation and suppresses subsequent production of proinflammatory cytokines such as RANTES, MCP-1, IL-6, and IL-8 in response to TNFα [312]. This may contribute to the renoprotective actions of Klotho, as evidenced in Klotho transgenic mice with renal mass reduction and ischemia reperfusion [111]. An anti-inflammatory effect of Klotho has also been observed in endothelial cells. Klotho attenuated the TNFα-induced monocyte adhesion to human umbilical vein endothelial cells, expression of adhesion molecules (ICAM-1 and VCAM-1) and NFκB activation both in vitro and ex vivo [158]. Klotho reverses the inhibition of eNOS phosphorylation by TNFα [158]. Klotho suppresses Nox2 protein expression and attenuates oxidative stress in vascular smooth muscle cells [294]. Klotho also suppresses retinoic-acid-inducible gene-I (RIG-I)-mediated inflammation. Mitochondrial dysfunction leading to excessive ROS or proinflammatory signals has been associated with aging [83]. RIG-I-signaling mediates senescence-associated inflammation in cooperation with MDA5 through activation of NFκB and interferon regulatory factors (IRFs). During kidney aging renal Klotho decreases and RIG-1 and IL-6 expression increases. Furthermore, Klotho-/- have early evidence of kidney and systemic inflammation characterized by increased serum IL-6 [143]. The intracellular form of Klotho interacts with the caspase recruitment domain (CARD) of RIG-I and inhibits RIG-I-induced expression of IL-6 and IL-8 both in vitro and in vivo, in endothelial cells and fibroblasts. The secreted form of Klotho did not affect RIG-I signaling [143].

Klotho can modulate mitochondrial oxidative stress and ameliorates renal injury. A single injection of recombinant Klotho protein improves AKI even when administered after injury in rats [112]. Adenovirus-

mediated Klotho gene transfer improved serum creatinine and renal morphological damage and reduced apoptosis induced by ischemia-reperfusion in rats via HSP70 [269;271]. Overexpression of Klotho in transgenic ICR-derived glomerulonephritis (ICGN) mice reduced oxidant stress and cell death [94].

Klotho genetic variants in humans

Genetic variants in Klotho have been described in humans. Single-nucleotide polymorphisms (SNPs) or loss of function mutations in the human klotho gene have been associated with age- and inflammation-related diseases, including osteoporosis, metabolic syndrome, severe tumoral calcinosis, stroke and coronary artery diseases, and predicted to influence overall survival. The KL-VS allele, one of the functional variants of Klotho, contains six sequence variants in complete linkage disequilibrium, two of which result in amino acid substitutions F352V and C370S. Homozygous elderly individuals were underrepresented in three distinct populations studied. Secreted levels of Klotho harboring V352 are reduced 6-fold, whereas extracellular levels of the S370 form are increased 2.9-fold and the V352/S370 double mutant exhibits an intermediate phenotype (1.6-fold increase) [10]. These results suggest that the KL-VS allele influences the trafficking and catalytic activity of Klotho [10]. A specific intronic Klotho variant (rs577912) is linked to survival in ESRD patients initiating chronic hemodialysis and therapy with active vitamin D may modify this risk [73]. The CC genotype of rs577912 associated to increased risk for mortality and was associated with a 16-21% lower Klotho expression. A homozygous missense mutation (H193R) in the Klotho gene resulted in hyperphosphatemia, hypercalcemia, increased FGF23 and PTH and severe tumoral calcinosis and carotid artery calcifications in a 13-year-old girl [116]. Expression and secretion of H193R KL were markedly reduced. However, there is little information on Klotho genotypes and inflammation in humans.

The way forward

In summary, evidence is emerging that supports a central role for Klotho and phosphate metabolism in the association of aging-related diseases and inflammation (Figure 1). The association between inflammation and aging is observed in the general population as well as in CKD patients. Systemic or local renal inflammation decreases kidney Klotho expression through specific cytokines such as TNFα or TWEAK and the canonical activation of the inflammatory transcription factor NFκB. Klotho itself has antioxidant and anti-inflammatory properties and inactivates NFκB. Klotho is a key regulator of phosphate balance and a role of phosphate in

aging has been shown. However, the potential relationship between phosphate and inflammation requires further clarification. A correct understanding of these interactions may lead to the design of novel therapeutic approaches to CKD and CKD-related inflammatory and aging features as well as to the inflammation/aging complex in general. Well known drugs with anti-inflammatory properties, such as angiotensin receptor antagonists and statins increase Klotho protein expression [138;174]. Anti-TWEAK antibodies are undergoing clinical trials in kidney disease. Certain phosphate binders decrease inflammatory components in humans [193;256]. Recently, higher FGF23 levels were reported to be independently associated with higher levels of inflammatory markers in patients with CKD and with significantly greater odds of severe inflammation [169]. There are several potential explanations for this observation that should be explored in detail. One of them is that low receptor levels (low Klotho) may promote ligand (FGF23) expression and that the observational association is a reflection of the association between low Klotho levels and inflammation. The discussion of the potential independent contribution of FGF23 to inflammation exceeds to scope of this review.

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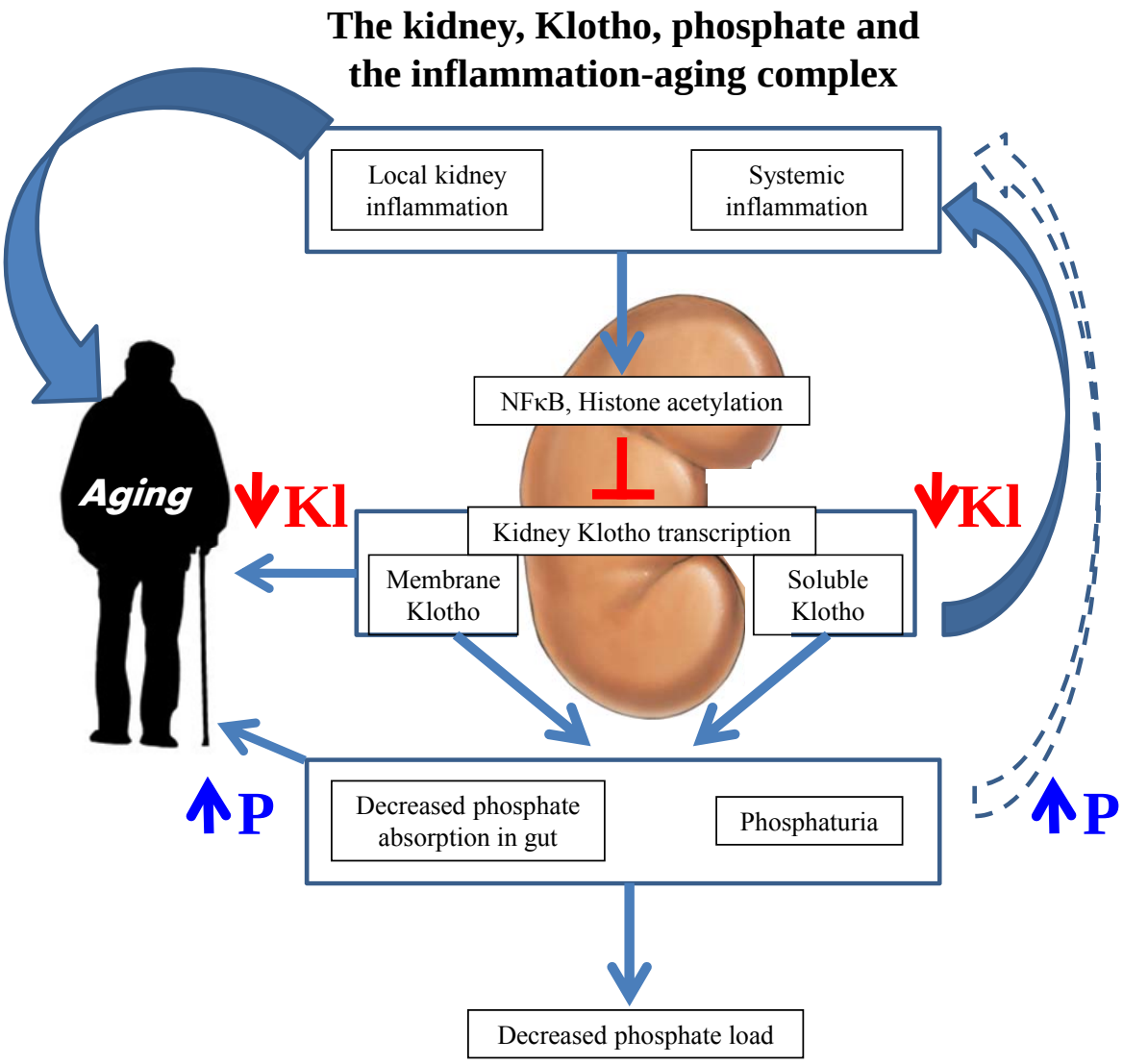

Legend to the figure

Central role of the kidney in modulating inflammation-induced aging through Klotho and regulation of phosphate metabolism. Kl: Klotho, P: phosphate.

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Acute kidney injury transcriptomics unveils a relationship between inflammation and ageing –inflamm-aging

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ABSTRACT

There are no pathophysiolgical therapeutic approaches to acute kidney injury (AKI) and the mortality remains high. In addition chronic kidney disease (CKD) predisposes to AKI and AKI contributes to progression of CKD. Recently a transcriptomics approach unveiled a relationship between AKI, inflammation and the regulation of ageing. A transcriptomics analysis of experimental AKI revealed increased kidney expression of Fn14 and transmembrane chemokine CXCL16, as well as a decreased expression of the kidney-secreted anti-ageing hormone Klotho. Fn14 is the receptor for tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily. In AKI kidneys there was a positive correlation between Fn14 and CXCL16 mRNA expression and an inverse correlation between Fn14 and Klotho mRNA. Tubular cells were the site of Fn14, CXCL16 and Klotho expression in vivo. Research on the relationships between these three molecules disclosed that TWEAK activation of Fn14 promoted inflammation through secretion of chemokines such as CXL16 in tubular cells in culture and in vivo. Furthermore, TWEAK activation of Fn14 decreased expression of Klotho mRNA and protein in culture and in vivo. Interestingly, both TWEAK activation of CXCL16 mRNA transcription and suppression of Klotho mRNA transcription were mediated by the NFκB transcription factor. In conclusion, TWEAK engagement of Fn14 is a central event promoting NFκB-mediated activation of inflammation pathways and suppression of anti-inflammatory/anti-ageing pathways. This information may influence future therapeutic approaches to AKI and inflamm-aging.

Key words: Acute kidney injury, aging, chronic kidney disease, inflammation, Klotho, TWEAK

Acute kidney injury and chronic kidney disease

Acute kidney injury (AKI) is a syndrome characterized by tubular injury and a sudden drop in glomerular filtration. Our current understanding of the pathophysiology of AKI is incomplete and this accounts for the lack of specific therapy. One key feature that has emerged in recent years is the close relationship between AKI and chronic kidney disease (CKD) [35]. Thus, CKD is the main risk factor for AKI and AKI contributes to progression of CKD. This suggests that AKI and CKD share pathogenic factors: from a pathogenic point of view CKD may be considered a low level, persistent AKI. Since pathogenic events are magnified in AKI and AKI has a shorter time course, AKI has advantages as a model for the identification and assessment of pathogenic factors. In this regard, proposed biomarkers of AKI are also altered in CKD, including Klotho [109;215].

Transcriptomics

High throughput techniques such as transcriptomics and proteomics, may help identify novel potential pathogenic factors, therapeutic targets and biomarkers in a non-biased way [16;237;265]. Transcriptomics is a high throughput technique that allows the identification of thousands of differentially expressed candidate genes. Such patterns of expression may themselves be used for diagnostic or prognostic purposes. Bioinformatics and biostatistics tools allow to manage thousands of genes simultaneous and help to prioritize molecules for further confirmatory studies. Novel therapeutic targets may be uncovered. We recently used a transcriptomic approach to identify new genes involved in AKI that could serve as biomarkers or therapeutic targets [121;179]. This approach has successfully identified new players in diabetic nephropathy such as the lethal cytokine TRAIL; the MIF receptor CD74 and the intracellular lethal protein BASP1 [149;239-241].

TWEAK and Fn14

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK, Apo3L, TNFSF12) is a member of the tumor necrosis factor superfamily (TNFSF) [40;247;248]. Other members of the family include TNF and Fas ligand, both of which play a key role in kidney injury [205;208;236]. TNFSF ligands bind to one or more members of the TNF receptor superfamily (TNFRSF) [19;147].

The human TWEAK gene encodes a type II transmembrane glycoprotein. The TWEAK C-terminal extracellular domain contains the TNF homology domain that mediates self-trimerization and receptor-binding [40].

The N-terminal intracellular domain contains several nuclear localization sequences (NLS) [14;40;52;162] and a furin recognition site, suggesting that TWEAK can be cleaved [25]. Most cells can express full-length membrane-anchored TWEAK (mTWEAK) and soluble TWEAK (sTWEAK) [25;188]. sTWEAK is formed by proteolysis of membrane TWEAK [40;188;300].

Both sTWEAK and mTWEAK bind and activate fibroblast growth factor-inducible-14 (Fn14, TWEAK receptor, TNFRSF12A, CD266) [25;69;226;298]. Fn14 was initially described in fibroblasts as a growth factor-regulated early response gene [167]. Fn14 is a type I transmembrane protein that when mature has 102-aa. Fn14 is the smallest member of TNFRSF. The intracellular Fn14 domain contains TNFR-associated factor (TRAF)-binding sites which activate signal cascade. Unlike TNF or Fas, Fn14 does not contain a death domain (DD) [26]. In addition, CD163 binds TWEAK and is thought to be a TWEAK scavenger receptor, since TWEAK-induced signaling through CD163 was not observed [24;181].

TWEAK has multiple functions with potential physiopathological relevance for kidney injury that depend on the microenvironment, the cell type and the cell state of activation. TWEAK can regulate cell proliferation, cell death, cell migration, cell differentiation, tissue regeneration, neoangiogenesis and inflammation [123;155;157;177;186;191;196;278;283;291]. TWEAK contributes to tissue injury in the central nervous system, liver, gut, the vasculature, skeletal muscle, heart and kidney [56;59;92;177;182;184;280].

In the kidney TWEAK actions have been extensively studied in tubular epithelium. TWEAK induces proliferation in non-stressed renal tubular cells [245] and apoptosis in tubular cells stressed by an inflammatory milieu [121;127]. Furthermore TWEAK activates both canonical and non-canonical NFκB transcription factor signaling [242;246;248;249]. Through these actions TWEAK promotes tubular injury in ischemic or toxic AKI [107;242] and kidney hyperplasia following unilateral nephrectomy [244]. Furthermore, TWEAK contributes to vascular injury and in CKD patients soluble TWEAK behaves as a biomarker of outcome, especially when interpreted in the context of systemic inflammation [17;31;211;308;309]. A recent transcriptomics analysis of kidney tissue in AKI confirmed highly upregulated levels of Fn14 mRNA (Figure 1). Regulation of the TWEAK/Fn14 system often takes places through upregulation of receptor expression and, thus, of cell sensitivity to TWEAK actions.

CXCL16

Chemokines are small cytokines formerly known as intercrines [78] that in the kidney tubulointerstitium may be expressed by both tubular cells and fibroblasts [80]. Chemokines promote leukocyte trafficking, growth and activation in inflammatory sites [225]. Chemokines promote kidney tubulointerstitial inflammation [45;180]. Leukocytes recruited by chemokines have a key role in kidney tubulointerstitial tissue injury during AKI and CKD [202;262]. Several chemokines were upregulated in the transcriptome of murine AKI [121]. Some of them, such as MCP-1 and Rantes, had already been studied [242]. These chemokines share with most chemokines their release as soluble mediators [45]. CX3CL1 (fractalkine) and CXCL16 (SR-PSOX) were also found upregulated in the murine AKI the transcriptome [121]. CX3CL1 and CXCL16 are the only two known membrane-anchored chemokines [153]. CX3CL1 and CXCL16 are synthesized as transmembrane molecules and, as such, have specific functions that may go beyond their chemokines role. In addition, they can be cleaved from the cell surface to release a soluble chemoattractant that behaves as a classical chemokine [153]. Fractalkine has been extensively studied in the context of kidney disease [133]. However, much less was known about CXCL16 and kidney injury. Furthermore, CXCL16 expression correlated more closely than CX3CL1 with Fn14 expression [121]. In addition, there is evidence that in humans urinary TWEAK and CXCL16 may be a potential diagnostic biomarkers of kidney diseases such as lupus nephritis [254;303]. TWEAK is known to regulate the expression of several chemokines. These peculiarities made a complete understanding of the relationship between TWEAK and CXCL16 regulation in kidney cells of particular interest.

CXCL16 was identified by different groups as a ligand for the CXC-chemokine receptor CXCR6 [164] and as a scavenger receptor for phosphatidylserine and oxidized low density lipoprotein (oxLDL) and therefore was also termed SR-PSOX [258]. Full length CXCL16 consists of an extracellular N-terminal chemokine domain, a glycosylated mucin-like stalk, a transmembrane-spanning region and a short cytoplasmic tail [15;164;213]. Like CX3CL1, CXCL16 potentially functions as both a soluble chemokine and a membrane-bound adhesion molecule [118;259]. CXCL16 regulated leukocyte chemotaxis, T cell recruitment and cell proliferation [33;48;55;96;113;252;315].

In the kidney, CXCL16 is constitutively expressed in human mesangial cells, podocytes and tubular cells [90;251;252]. There is evidence for differential regulation of CXCL16 expression in glomeruli or different tubular segments and in tubular injury of diverse etiology. CXCL16 expression is increased in various animal models of kidney injury and human nephropathies [75;90;251;252;302;303].

Glomerular CXCL16 expression is increased in human membranous nephropathy [90]. Glomerular and tubular CXCL16 was also increased in lupus mice and in anti-GBM nephritis [75;302;303]. Functional studies suggest that CXCL16 promotes progression of damage in experimental glomerulonephritis [75]. CXCL16 blockade significantly decreased monocyte/macrophage infiltration and glomerular and tubular injury [75;302]. In this regard, besides effects on leukocytes, CXCL16 has direct actions on glomerular cells. Podocyte CXCL16 may regulate the uptake of oxLDL [90], while mesangial cell CXCL16 promotes mesangial cell migration and proliferation [252].

In human allograft AKI, CXCL16 expression was increased focally in the apical side of tubules [251]. By contrast, a low tubular CXCL16 expression was observed in interstitial rejection that was attributed to increases CXCL16 shedding. Thus, remnant CXCL16 was located to the basolateral membrane and surrounded by T cell infiltrates. In experimental toxic AKI, both prominent apical and basolateral CXCL16 expression were noted [121]. Thus, other tubular cells, the interstitium and the tubular lumen were exposed to CXCL16 derived from tubular cells. Interestingly, both patterns did not overlap in many tubules.

No in vivo functional studies of CXCL16 targeting in tubulointerstitial kidney disease have been reported. In cell culture CXCL16 did not induce murine tubular epithelial cell proliferation or apoptosis, either alone or in combination with TWEAK [121]. However, CXCL16 had a proinflammatory effect and increased TWEAK-induced gene expression of ICAM-1, MCP-1 and RANTES. In this regard, tubular cells expressed the CXCR6 receptor [121].

In cultured glomerular cells CXCL16 is upregulated by TNF-α and IFN-γ [90;251;252]. IFN-γ increased CXCL16 expression in cultured primary thick ascending limb cells and early distal tubular cells [251]. TWEAK is a novel regulator of CXCL16 expression in tubular epithelial cells [121]. TWEAK promoted CXCL16 expression through the canonical NFκB pathway in cultured tubular cells [121]. Moreover, TWEAK increased renal CXCL16 expression and interstitial CD3 positive lymphocytes. Since neutralization of TWEAK decreased CXCL16 and CD3 lymphocyte infiltration in experimental AKI, TWEAK-induced CXCL16 expression may contribute to T cell recruitment and collaborate with TWEAK in promoting inflammation.

Klotho

Klotho is a protein with anti-aging properties which is highly expressed in tubular renal cells [135;137]. Klotho is a single-pass transmembrane protein. The extracellular domain of Klotho may be proteolytically processed by ADAM10/17 and secreted. In addition, alternative splicing may give rise to a soluble secreted isoform [108].

Trasmembrane Klotho binds to multiple fibroblast growth factor (FGF) receptors conferring them specific and high affinity for FGF23. FGF23 is a bone-derived hormone that regulates phosphate homeostasis and vitamin D metabolism. Thus, the main known function of Klotho is regulation of phosphate metabolism and evidence from mice in which phosphate was manipulated genetically or through diet suggests that aberrant phosphate homeostasis is a key contributor to the accelerated aging syndrome of Klotho -/- mice [197]. Klotho also protects cells and tissues from oxidative stress and has anti-inflammatory properties through modulation of NF-κB signaling [311].

Klotho is downregulated during kidney diseases, such as long-term hypertension, diabetes mellitus, CKD [5],and in experimental AKI induced by ischemia-reperfusion or a folic acid overdose [179;272] . In addition kidney Klotho was decreased in the course of systemic inflammation caused by inflammatory bowel disease and a neutralizing anti-TNF antibody attenuated bowel inflammation and reversed the repression of kidney Klotho expression [275]. Consistent with these data, Klotho was downregulated in the transcriptome of murine AKI and Klotho expression was inversely correlated with Fn14 expression, suggesting that TWEAK, like TNF, may regulate Klotho expression. The reduction of kidney Klotho during nephrotoxic AKI persisted beyond recovery of renal function and was associated with decreased circulating Klotho. The persistent decrease in Klotho might be related to the increased mortality of AKI patients following recovery from AKI. Since Klotho may be nephroprotective [94;112;268;272], the persistent decrease in Klotho might also predispose to progression of CKD. However, these hypotheses await formal confirmation.

In nephrotoxic AKI, Klotho expression and renal function were preserved by TWEAK targeting thus identifying a potential regulator of Klotho expression in cultured cells [179]. Indeed, in cultured tubular cells of proximal origin TWEAK and TNF promoted the NFκB-dependent downregulation of Klotho expression [179]. TWEAK and TNF activate the canonical pathway for NFκB activation, but only TWEAK activates the non-canonical pathway [246;248]. The reported downregulation of Klotho by TNF [179;275] and the time course of Klotho mRNA downregulation, that is already observed at 3h, suggest activation of the canonical NFκB pathway. Indeed, RelA was necessary for TWEAK- and TNF-induced Klotho repression. For the first time it was observed TWEAK downregulates NFκB-mediated gene expression. Regulation of NFκB activation function is controlled through different mechanism, such as interaction of the p65/RelA subunit with histone deacetylase (HDAC) corepresor proteins [1;28;145]. In this regard, HDAC inhibitors prevented repression of Klotho induced by TWEAK or TNF. In addition, recruitment of

NFκB to chromatin is regulated in a promoter-specific manner. TWEAK induced histone H3 and H4 deacetylation at the murine Klotho promoter in renal tubular cells.

Interaction between inflammation and ageing: NFκB

From the above mentioned studies the NFκB emerges as a family of pleiotropic transcription factors with a key role at the interface between inflammation and ageing [13;98;217;249;290]. This notion had been advanced before by proponents of the inflamma-aging concept [235]. Inflamm-aging describes the age-related increase in the systemic pro-inflammatory status of humans [72].

A wide range of stimuli relevant to tissue injury activate NFκB, including cytokines, growth factors, immune mediators, proteinuria and genotoxic or mechanical stretch[37;87]. Activation of NFκB can proceed through classical/canonical, alternative/non-canonical NFκB and hybrid pathways[21;98;290]. Classical NFκB activation is usually a rapid and transient response to a wide range of stimuli. Under basal conditions NFκB is inactive in the cytosol because it is bound to inhibitory IκB proteins. Activating stimuli activate the inhibitor of κB kinases (IKK), which phosphorylate IκBs, marking them for degradation by the proteasome. Degradation of IκB releases and activates NFκB dimers, such as those containing RelA. RelA containing dimers then migrate to the nucleus where they bind to κB DNA sequences in promoters and enhancers of target genes. In general canonical NFκB promote the transcription and expression of proinflammatory genes, as observed for CXCL16 in TWEAK-stimulated tubular cells. There are several negative feed-back mechanisms. Thus, suppressors of cytokine signaling (SOCS)-1 promotes the ubiquitination and proteasomal degradation of RelA-containing dimers, thus quenching the NFκB response [77]. The SOCS1 overexpression decreases inflammation in experimental DN [212].

As a result of NFκB integration of stimulus information it may both induce or repress individual gene transcription [104]. However, the fact that NFκB can function as a repressor of gene expression is less well-known. Gene expression repression by NFκB may suppress the inflammatory response by recruiting inhibitory components of the NFκB system. Thus, antiinflammatory cytokines, such as IL-10 promote synthesis of nuclear located atypical IκB proteins B-cell lymphoma 3 (BCL-3), IκBζ and IκBNS, which bind to DNA-bound NFκB dimers and may repress transcription of inflammatory genes [77]. In addition repression of gene expression by NFκB has been implicated in sepsis-induced downregulation of kidney aquaporin/V2 receptor and may have a role in resolution of inflammation [102;214]. However, classical NFκB dimers containing RelA may also downregulate Klotho

mRNA and Klotho-dependent anti-inflammatory and ageing pathways, as observed for TWEAK and TNF, and, thus, promote further injury in and outside the kidney.

Conclusions

In summary, transcriptomics of AKI tissue has identified TWEAK as a novel regulator of CXCL16 expression in renal tubular cells through activation of the RelA NFκB transcription factor. In addition, TWEAK, like TNFα, downregulated Klotho in renal tubular cells through a similar NFκB RelA-dependent mechanism. Since Klotho has anti-ageing and anti-inflammatory properties, these findings may have therapeutic implications in kidney injury and also for inflammation-associated premature aging. Thus targeting either TWEAK, through neutralizing anti-TWEAK antibodies currently undergoing clinical trials in lupus nephritis, or targeting NFκB, may potentially limit inflammation and the adverse consequences of inflammation on ageing (inflamm-aging).

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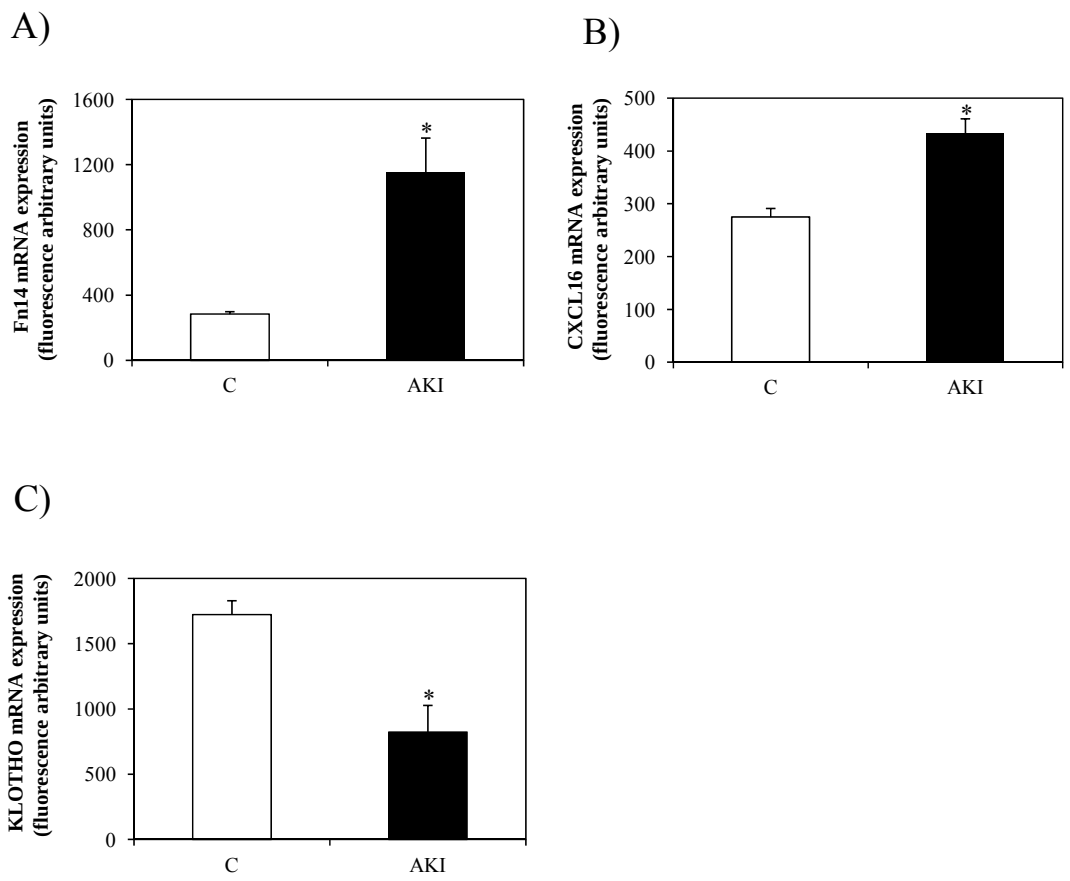

Figure 1. Gene expression for representative mediators of inflammation and ageing in experimental acute kidney injury (AKI): transcriptomics results of kidney tissue. A) Fn14 mRNA. B) CXCL16 mRNA. C) KLOTHO mRNA. * $p<0.005$ vs vehicle-injected controls (C). Data expressed as mean (SEM).

Key concepts

1. Tissue transcriptomics allows the non-biased analysis of gene expression and identification of potential novel therapeutic targets in tissue injury

2. Acute kidney injury transcriptomics identified the simultaneous upregulation of inflammatory genes such as the TWEAK receptor FN14 and chemokines like CXCL16 and the downregulation of anti-inflammatory/anti-ageing genes such as Klotho

3. TWEAK stimulation of tubular cells in culture reproduced the findings in AKI.

4. The transcription factor NF-κB appears to be the key to both upregulation of proinflammatory genes and downregulation of Klotho in response to TWEAK

5. Thus either targeting TWEAK, through neutralizing anti-TWEAK antibodies currently undergoing clinical trials in lupus nephritis, or targeting NF-κB, may potentially limit inflammation and the adverse consequences of inflammation on ageing (inflamm-aging)